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(54) Title: CELL DEATH AGONISTS

(57) Abstract

Small polypeptides and peptides of 5 to 50 amino acids having cell death agonist activity are provided. The polypeptides are at least 9 amino acids in length and contain the BH3 domain of a pro-apoptotic BCL-2 family member. The peptides contain 5 to 8 amino acids from the BH3 domain. Methods of promoting apoptosis with these cell death agonist polypeptides and peptides and their encoding polynucleotides are also provided.

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CELL DEATH AGONISTS

Cross-Reference to Related Applications

This application claims the benefit of, and incorporates herein by reference, the U.S. Provisional Application entitled "BH3 Domain of Bad is Required for Heterodimerization with BCL-X_L and Pro-Apoptotic Activity", which was filed September 26, 1997 as Attorney Docket No. 6029-1985.

Reference to Government Grant

This invention was made with government support under Grant Number R01 #50239. The government has certain rights in this invention.

Background of the Invention

(1) Field of the Invention

This invention relates generally to the regulation of apoptosis and to compounds which regulate apoptosis, and more particularly, to a novel cell death agonist.

(2) Description of the Related Art

Programmed cell death, referred to as apoptosis, plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms (Raff, *Nature 356*:397-400, 1992). Genetic and molecular analysis from nematodes to humans has indicated that the apoptotic pathway of cellular suicide is highly conserved (Hengartner and Horvitz, *Cell 76*:1107-1114, 1994) In addition to being essential for normal development and maintenance, apoptosis is important in the defense against viral infection and in preventing the emergence of cancer.

The BCL-2 family of proteins constitutes an intracellular checkpoint of apoptosis. The founding member of this family is the apoptosis-inhibiting protein encoded by the *bcl-2* protooncogene which was initially isolated from a follicular lymphoma (Bakhshi et al., *Cell 41*:889-906, 1985; Tsujimoto et al, *Science 229*:1390-1393, 1985; Cleary and Sklar, *Proc Natl Acad Sci USA 82*:7439-7443, 1985). The BCL-2 protein is a 25 kD, integral membrane protein localized to intracellular membranes including mitochondria. This factor extends survival in many different cell types by inhibiting apoptosis elicited by a variety of death-inducing stimuli (Korsmeyer, *Blood 80*:879-886, 1992).

The family of BCL-2-related proteins is comprised of both anti-apoptotic and pro-apoptotic members that function in a distal apoptotic pathway common to all multi-cellular organisms. It has been suggested that the ratio of anti-apoptotic (BCL-2, BCL-X_L, MCL-1 and A1) to pro-apoptotic (BAX, BAK, BCL-X_S, BAD, BIK and BID) molecules dictates whether a cell will respond to a proximal apoptotic stimulus. (Oltvai et al., *Cell 74*:609-619, 1993; Farrow, et al., *Curr. Opin. Gen. Dev. 6*: 45-49, 1996). Because members of this family can form both homodimers and heterodimers, the latter often between anti- and pro-apoptotic polypeptides, the balance

of these homodimers and heterodimers could play a role in regulating apoptosis (Oltvai and Korsmeyer, *Cell 79*:189-192, 1994).

Members of the BCL-2 family have been defined by sequence homology that is largely based upon conserved motifs termed BCL-Homology domains. (Yin et al, *Nature 369*:321-323, 1994). BCL-Homology domains 1 and 2 (BH1 and BH2) have been shown to be important in dimerization and in modulating apoptosis (Yin et al., *supra*). A third homology region, BH3, has been found in some family members and shown to be important in dimerization as well as promoting apoptosis (Boyd et al., *Oncogene 11*:1921-1928; Chittenden et al., *Embo J 14*:5589-5596, 1995). BH4, the most recently identified homology domain, is present near the amino terminal end of some pro-apoptotic family members (Farrow et al., *supra*).

The BH3 domain may play a role in the promotion of death by full-length pro-apoptotic family members, although BAD was not heretofore known to contain a BH3 domain. For example, the pro-apoptotic family member BCL-X_S, which is translated from an alternatively spliced version of the mRNA encoding BCL-X_L, contains BH3 and BH4 domains, but lacks BH1 and BH2 domains. BCL-X_S inhibits the ability of BCL-2 to enhance the survival of growth-factor deprived cells (Boise et al. *Cell 74*:597-608, 1993). BIK and BID are other death promoting BCL-2 family members having a BH3 but not BH1 or BH2 domains and which also lack a BH4 domain (Boyd et al., *Oncogene 11*:1921-1928, 1995; Wang et al., *Nature 379*:554-556, 1996).

Deletion analysis has indicated that the BH3 domain of the pro-apoptotic family members BAK, BAX, and BIK is required for them to heterodimerize with BCL-X_L or BCL-2 and also to promote cell death (Chittenden et al., *Embo J 14*:5589-5596, 1995; Zha et al., *supra*). For example, a significant loss of viability was observed in

cells transiently transfected with a plasmid expressing a 51 amino acid BAK polypeptide which contained BH3 but lacked BH1 and BH2 (Chittenden et al., *supra*). However, a BH3-containing 46 amino acid fragment of BAK, which bound to BCL-X_L both *in vitro* and in transfected cells, was reported to exhibit no cell killing activity unless the BAK hydrophobic tail element was attached (Chittenden et al., *supra*).

Other mutagenesis studies revealed that pro-apoptotic BID also interacts with BCL-2, BCL-X_L, and BAX through its BH3 domain and indicated that the corresponding binding site on these partner proteins is the BH1 domain, and perhaps also the BH2 domain (Wang et al., *supra*.) These data in combination with the predicted three-dimensional structures of BCL-2 and BAX, which are similar to the solved structure of BCL-X_L (Muchmore et al., *Nature 381*:335-341, 1996), were suggested to support a hypothesis that a BH3-BH1 mediated interaction between BID and a partner protein would occur by binding of the amphipathic α-helix of BID's BH3 domain to the exposed hydrophobic cleft contributed by the BH1 domain of the partner protein (Wang et al., *supra*).

A recent article described the three-dimensional structure of a complex between full-length BCL-X_L and a 16 amino acid Bak peptide (BAK 72-87) containing the BH3 domain (Sattler et al., *Science 175*:983-986, 1997). The BAK peptide, which is a random coil in solution, forms an α helix upon binding in a hydrophobic cleft formed by the BH1, BH2, and BH3 regions of BCL-X_L, with certain hydrophobic side chains of the BAK peptide (Val^{74}, Leu^{78}, and Ile^{81}) pointing into the cleft and certain charged side chains of the peptide (Arg^{76}, Asp^{83}, and Asp^{84}) being close to oppositely charged residues of BCL-X_L. Smaller BAK peptides from this region, including an 11mer peptide corresponding to BAK residues 77 to 87, reportedly did not bind to BCL-X_L.

However, BH3-BH1 binding may not be involved in all interactions between BCL-2 related proteins. For example, pro-apoptotic BIK and BCL-X_s, both of which lack the BH1 and BH2 domains, have been shown to interact (Boyd et al., *supra*). In addition, it has been demonstrated that BAX does not require BH1 or BH2 to homodimerize (Zha et al., *supra*).

Some disease conditions are believed to be related to the development of a defective down-regulation of apoptosis in the affected cells. For example, neoplasias may result, at least in part, from an apoptosis-resistant state in which cell proliferation signals inappropriately exceed cell death signals. Furthermore, some DNA viruses such as Epstein-Barr virus, African swine fever virus and adenovirus, parasitize the host cellular machinery to drive their own replication and at the same time modulate apoptosis to repress cell death and allow the target cell to reproduce the virus. Moreover, certain disease conditions such as lymphoproliferative conditions, cancer including drug resistant cancer, arthritis, inflammation, autoimmune diseases and the like may result from a down regulation of cell death regulation. In such disease conditions it would be desirable to promote apoptotic mechanisms.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

Summary of the Invention

In accordance with the present invention, it has been discovered that relatively short polypeptides including a BH3 domain derived from a pro-apoptotic

member of the BCL-2 family can promote apoptosis. Such polypeptides are shorter than the full length of the family member from which it is derived. The term "pro-apoptotic BCL-2 family member" refers to any polypeptide having a BH3 domain as defined herein and having the ability to promote cell death in one or more of the assays described herein. Pro-apoptotic family members include BAD, BAK, BAX, BID, and BIK.

The present invention is based on the discovery reported herein (1) that BAD (Bcl-2 Associated cell Death promoter) has a BH3 domain which is essential for apoptotic function and (2) that the BH3 domain of any pro-apoptotic member of the BCL-2 family is sufficient to promote apoptosis. In particular, the inventor has discovered that small polypeptides of 50 or fewer amino acids comprising the 9 amino acid BH3 domain have significant death agonist activity when administered to cells. This discovery was unexpected because it was not previously known that all BCL-2 pro-apoptotic family members contain a BH3 domain, nor was it known that a polypeptide containing the BH3 domain of any pro-apoptotic member is sufficient to promote apoptosis.

Accordingly, one aspect of the present invention provides a polypeptide containing a bcl-homology domain 3 (BH3 polypeptide) of from about 9 to about 50 contiguous amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The BH3 domain comprises a nine amino acid sequence as set forth in SEQ ID NO:40 (Leu-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Asp-Xaa_5-Xaa_6-Xaa_7, wherein Xaa_1 is Arg or Ala, Xaa_2 is Arg, Ile, Leu, Lys, Gln or Cys, Xaa_3 is Met, Ile or Val, Xaa_4 is Ser or Gly, Xaa_5 is Glu, Asp or Ser, Xaa_6 is Phe, Ile, Leu or Met, and Xaa_7 is Val, Glu, Asn or Asp), or a conservatively substituted variant thereof, and which is identified more particularly by homology to the sequences shown in FIG. 1 (SEQ ID NO:1-9). In preferred

embodiments, the BH3 domain is identical to or is a conservatively substituted variant of a BH3 domain from a human or murine BAD, BAK, BAX, BID, or BIK polypeptide. In one embodiment, the BH3 polypeptide is operably linked to a cell penetrating agent.

Another aspect of the invention provides a BH3 domain peptide having death agonist activity which comprises between about five to eight contiguous amino acids from the BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof.

Yet another aspect of the invention provides polynucleotides encoding a BH3 polypeptide of no more than 50 amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The invention also provides polynucleotides encoding BH3 domain peptides of about five to eight contiguous amino acids from SEQ ID NO:40, or a conservatively substituted variant thereof. These polynucleotide may be used to transfect a target cell for expression of the BH3 polypeptide to promote death of the target cell.

In other embodiments, the present invention provides a method for promoting apoptosis in a target cell comprising administering to the cell a death-promoting amount of a BH3 polypeptide or a BH3 domain peptide. The BH3 polypeptide comprises no more than 50 contiguous amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member, while the BH3 domain peptide has cell death agonist activity and comprises five to eight contiguous amino acids of the BH3 domain. In one embodiment, the BH3 polypeptide or BH3 domain peptide is operably linked to a cell-penetrating agent which improves entry of the BH3 polypeptide into the cell. Alternatively, the BH3 polypeptide or BH3 domain peptide can be administered to the target cell by transfecting

the cell with an expression vector which comprises a polynucleotide encoding the BH3 polypeptide or BH3 domain peptide.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of new BH3 polypeptides which are relatively short in length and which possess cell death agonist activity; the provision of peptides from the BH3 domain, the provision of polynucleotides encoding these polypeptides and peptides; the provision of BH3 polypeptide compositions and peptide compositions having cell death agonist activity and which can be readily delivered intracellularly to produce a death agonist activity; and the provision of a method for promoting death of a target cell with these compositions.

Brief Description of the Drawings

Figure 1 illustrates the amino acid sequences of the BH3 domains from human (h) and murine (m) BAD, BAK, BAX, BIK, and BID (SEQ ID NO:1-9);

Figure 2 illustrates the structures of BCL-2 family members showing the locations of the homology domains relative to the N-terminus as BH4, BH3, BH1, and BH2, with TM representing the hydrophobic transmembrane C-terminal tail present in most members;

Figure 3 illustrates that BAD has a BH1/BH3 region that is required for cell death and heterodimerization with BCL-2 showing (A) a map of a nested set of BAD deletion mutants indicating retained amino acids and the position of the BH1/BH3 and BH2 domains and (B) the binding of P^{32}-labeled GST-BCL-2 to these BAD deletion mutants transferred to nitrocellulose (upper panel) from a SDS-PAGE gel (lower panel);

Figure 4 illustrates aligned partial sequences of human and murine BAD, BAK, BAX, BID, and BIK (SEQ ID

NO:10-18) showing the sequence homology within BH3 domains (underlined) with identical amino acids boxed;

Figure 5 illustrates the predicted three-dimensional amphipathic α-helix structure of the BAD BH3 domain showing views of the hydrophobic surface (left) and polar surface (right) with the locations of the hydrophobic and polar amino acids forming each surface identified;

Figure 6 illustrates that the BAD BH1/BH3 domain is essential for pro-apoptotic function showing (A) the structure of BAD deletion mutants indicating retained amino acids and positions of the BH1/BH3 and BH2 domains, (B) the apoptosis-promoting activity of these BAD deletion mutants as measured by transient co-transfection with a luciferase reporter vector into BAD-deficient murine embryonic fibroblasts, and (C) the BCL-2 or BCL-X_L binding ability of these BAD deletion mutants in an *in vitro* binding assay;

Figure 7 illustrates the effect of BAD BH3 mutations on heterodimerization of BAD with BCL-2 or BCL-X_L showing (A) ^{35}S-labeled wild-type (WT) and mutant BAD proteins substituted with alanine at positions Gly 148 (G148A), Arg 149 (R149A), or Leu151 (L151A) produced by *in vitro* transcription-translation (IVTT) and the amount of these ^{35}S-labeled BAD proteins that were captured by GST-BCL-2 or GST-BCL-X_L bound to GSH-agarose beads in an *in vitro* binding assay, (B) a Western blot of lysates from FL5.12 BCL-X_L cells stably expressing wild-type or mutant forms of BAD probed with an anti-BAD antibody (upper panel) or an anti-BCL-X_L antibody (lower panel), and (C) a western blot analysis of levels of wild-type and mutant BAD proteins in total cell lysates (lysates), in BCL-X_L co-immunoprecipitates from the lysates (IPαBCL-X_L), and in the supernatant following removal of BCL-X_L/BAD complexes (Sup);

Figure 8 illustrates the effects of mutations in BAD BH1 and BH3 domains on intracellular distribution and death promoting activity, showing (A) proteins detected by anti-BAD Ab probing of a Western blot of crude membrane and cytosol fractions from FL5.12BCL-X_L cells expressing WT or mutant BAD proteins, (B) Western blot detection of proteins associated with WT and mutant BAD in the cytosolic fraction as determined by co-immunoprecipitation with anti-BAD mAb 2G11, and (C) a graph of viability of FL5.12BCL-X_L cells expressing WT or mutant BAD proteins as determined by propidium iodine exclusion at 24 hr., 48 hr., and 72 hr. after withdrawal of interleukin-3;

Figure 9 illustrates the effect of BCL-2 BH1, BH2, and BH3 mutations on heterodimerization of BCL-2 with BAD showing ^{35}S-labeled wild-type (WT) and mutant BCL-2 proteins substituted with alanine at positions Gly 145 (G145A), Trp 188 (W188A), or Leu97 (L97A) produced by *in vitro* transcription-translation (IVTT) and the amount of these ^{35}S-labeled BCL-2 proteins that were captured by GST-BAD bound to GSH-agarose beads in an *in vitro* binding assay;

Figure 10 illustrates (A) the BH3 domain of murine BID, represented with two upstream and two downstream amino acids (SEQ ID NOS:19) and a schematic representation of mutations introduced into BID (SEQ ID NOS:20-23) and (B) *in vitro* binding of BCL-2 or BAX with GST-BID or BID mutants;

Figure 11 illustrates (A) the viability of FL5.12-Bcl-2 clones expressing wild type or BH3-domain mutant BID, (B) Western blot showing BID expression and (C) Western blot showing association of wild type or BH3-domain mutant BID with BCL-2 and BAX (Lane 1: FL5.12-Bcl-2/Hygro.1; Lane 2: FL5.12-Bcl-2/Bid-8; Lane 3: FL5.12-Bcl-2/BidmIII-1.15; Lane 4: FL5.12-Bcl-2/BidmIII-2.10;

Lane 5: FL5.12-Bcl-2/BidmIII-3.1; Lane 6: FL5.12-Bcl-2/BidmIII-4.1);

Figure 12 illustrates (A) the viability of Jurkat cells expressing wild type and BH3-domain mutant BID; (B) Western blot showing levels of BID polypeptides; and (C) viability measured in luciferase activity in Rat-1 fibroblasts co-transfected with the luciferase reporter gene and with *bcl-2*, *bcl-2* along with *bid*, and with wild type and BH3-domain mutant *bid*;

Figure 13 illustrates the death-promoting activity of full-length BAX BH3-domain mutants showing (A) the location of substitution mutations made in or near the BH3 domain (SEQ ID NOS:24-29), (B) the luciferase activity in Rat-1 cells co-transfected with a luciferase reporter gene and a recombinant pcDNA3 vector encoding wild-type BAX, a BAX BH3-domain mutant or wild-type BCL-2, and (C) the amount of luciferase activity in Rat-1 cells co-transfected with both BCL-2 and a wild-type or BH3-domain BAX mutant.

Figure 14 illustrates various regions of (A) BAX and (B) BID proteins tested for death-promoting activity when encoded by expression vectors transiently transfected into cells;

Figure 15 illustrates the death-promoting ability of various BAX and BID regions showing (A) and (B) the amount of luciferase expression in Rat-1 cells at 20 hours after co-transfection with or without a pcDNA3 vector encoding BCL-2 and with recombinant pcDNA3 vectors encoding the (A) BAX regions or (B) BID regions, and (C) the amount of luciferase expression in Rat-1 cells grown in the presence or absence of the caspase inhibitor z-VAD-fmk at 20 hrs following transfection with recombinant pcDNA3 vectors encoding the indicated BAX and BID regions;

Figure 16 illustrates the effect of BH3 polypeptides on nuclear morphology of cells showing

photographs of Rat-1 cells transfected with (A) BAX WT, (B) BAX 53-104, (C) BID WT, or (D) BID 74-128 and stained with the DNA dye Hoechest 33342;

Figure 17 illustrates the death-promoting ability of Tat-BH3 peptides showing (A) the sequences of synthetic peptides consisting of an 11 amino acid sequence from the HIV I Tat protein (SEQ ID NO:55) linked to BAX or BID amino acid sequences containing a wild-type or mutant (m) BH3 domain and varying lengths of wild-type flanking region (SEQ ID NOS:30-39) and (B) the viability of 2B4 cells determined by trypan blue dye exclusion at four hours after no treatment or treatment with 100 μM of the Tat peptide or one of the Tat-BH3 peptides shown in (A);

Figure 18 illustrates the kinetics and dose-response relationship of cell death induced by Tat-BH3 peptides containing a wild-type or mutant BH3 domain from BAX or BID showing the viability of 2B4 cells determined by trypan blue dye exclusion (A) at different times following no treatment or treatment with 100 μM of the designated Tat-BH3 peptide and (B) at two hours after treatment with different doses of the Tat-BH3 peptide;

Figure 19 illustrates the effect of BCL-2 and z-VAD-fmk on cell death induced by Tat-BH3 peptides showing (A) the viability of 2B4 cells overexpressing BCL-2 or the vector alone (neo) determined by trypan blue dye exclusion at two hours after no treatment or treatment with Tat-BAX(57-71) or Tat-BID(81-100) at 100 μM concentration in the presence or absence of 200 μM z-VAD-fmk and (B) the percentage of these cells with subdiploid DNA (<2n) as determined by PI staining followed by flow cytometry;

Figure 20 illustrates the effect of Tat-BH3 peptides on cell morphology showing photographs of Jurkat cells treated for two hours with 100 μM of (A, B) Tat-

BAX(57-71) or (C, D) Tat-BID(81-120), stained with the DNA dye Hoescht 33342 and examined by (A, C) phase contrast light microscopy or (B, D) fluorescent microscopy;

Figure 21 illustrates the amino acid sequences for murine and human pro-apoptotic family members showing (A) full-length murine BAD and partial human BAD sequences (SEQ ID NOS:41 and 42), with conservative amino acid substitutions indicated by a dot (.), (B) full-length murine and human BAK sequences (SEQ ID NOS: 43 and 44), (C) full-length murine and human BAX sequences (SEQ ID NOS: 45 and 46), (D) full-length murine and human BID sequences (SEQ ID NOS: 47 and 48), with conservative amino acid substitutions indicated by a dot(.), and (E) full-length human BIK (SEQ ID NO: 49); and

Figure 22 illustrates the nucleotide sequences of human cDNAs showing (A) a partial bad cDNA (SEQ ID NO:50) which encodes a BH3-containing BAD polypeptide, (B) a bak cDNA (SEQ ID NO:51) encoding full-length BAK, (C) a bax cDNA (SEQ ID NO:52) encoding full-length BAX, (D) a bid cDNA (SEQ ID NO:53) encoding full-length BID, and (E) a bik cDNA (SEQ ID NO:54) encoding full-length BIK.

Description of the Preferred Embodiments

The present invention is based, in part, upon the unexpected discovery that BAD, like all other known pro-apoptotic members of the BCL-2 family, has a BH3 domain and that this domain is necessary for BAD's death agonist activity. This discovery was unexpected because BAD has been previously reported as containing only BH1 and BH2 domains in common with BCL-2 family members. Yang et al., *Cell 80*:285-291, 1995, incorporated herein by reference. Moreover, unlike all other BH1- and BH2-containing family members, in which the BH3 domain is located N-terminal to the BH1 domain (Fig. 2), the BH3 domain of BAD is located between the BH1 and BH2 domains

and indeed partially overlaps the C-terminal portion of the BH1 domain (Fig. 2). The heretofore unrecognized presence of a BH3 domain in all known pro-apoptotic members of the BCL-2 family along with the herein described death inducing activity of short BH3-containing polypeptides establishes for the first time that the BH3 domain is sufficient for inducing cell death. It is also believed that peptides as short as five amino acids from the BH3 domain will also have death agonist activity.

Therefore, the present invention provides a BH3 polypeptide of at least 9 and no more than 50 amino acids comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The BH3 domain comprises a nine amino acid sequence as set forth in SEQ ID NO:40: Leu-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Asp-Xaa_5-Xaa_6-Xaa_7, wherein Xaa_1 is Arg or Ala, Xaa_2 is Arg, Ile, Leu, Lys, Gln or Cys, Xaa_3 is Met, Ile or Val, Xaa_4 is Ser or Gly, Xaa_5 is Glu, Asp or Ser, Xaa_6 is Phe, Ile, Leu or Met, and Xaa_7 is Val, Glu, Asn or Asp; or a conservatively substituted variant thereof.

A conservatively substituted variant of SEQ ID NO:40 is an amino acid sequence having identity to or conservative amino acid substitutions at any of the nine positions of SEQ ID NO:42. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids which have neutral and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic side chains (G, A, V, L, and I); another grouping is

those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions are: R-K; E-D, Y-F, L-M; V-I, and Q-H. A conservatively substituted variant of SEQ ID NO:40 also includes the amino acid sequence of a BH3 domain identified in any subsequently discovered BCL-2 family member which has cell death agonist activity.

In preferred embodiments, the BH3 domain is from a mammalian pro-apoptotic BCL-2 family member. More preferably, the BH3 domain is from murine or human BAD, (FIG. 21A) BAK (FIG. 21B), BAX (FIG. 21C), BID (FIG. 21D), or human BIK (FIG. 21E) and comprises an amino acid sequence as set forth in any of SEQ ID NO:1-9 (FIG 1). Most preferably, the BH3 domain is a human amino acid sequence as set forth in any of SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

In addition to the BH3 domain of nine contiguous amino acids, the BH3 polypeptide can comprise at least one and up to 41 additional amino acids which flank the BH3 domain or which are contiguous to the N-terminal or C-terminal amino acids of the BH3 domain. Preferably, the BH3 polypeptide comprises between at least about 9 and about 50 contiguous amino acids and can have a length of any number between 9 and 50. More preferably, the BH3 polypeptide comprises at least 11 amino acids and even more preferably, the BH3 polypeptide is between at least 15 and 24 contiguous amino acids in length.

The amino acid sequence of the BH3 polypeptide can be any sequence provided that it includes a BH3 domain as defined above and that the polypeptide has cell death agonist activity. The term "cell death agonist activity"

is intended to mean that the BH3 polypeptide is capable of inducing cell death in a similar fashion, although not necessarily to the same degree, as the polypeptides particularly exemplified herein. The cell death agonist activity of a polypeptide can be readily examined using one of the cell assays described herein. It is believed that the amino acid sequence of the BH3 polypeptide should be one which folds in such a manner that the BH3 domain is exposed on the surface of the surface of the polypeptide.

Preferably, the BH3 polypeptide comprises a BH3-containing sequence of between at least 9 and 50 contiguous amino acids from a pro-apoptotic BCL-2 family member. Even more preferably, the BH3-containing sequence is from one of the human polypeptide sequences shown in Figure 21: BAD (SEQ ID NO:41), BAK (SEQ ID NO:42), BAX (SEQ ID NO:43), BID (SEQ ID NO:44) or BIK (SEQ ID NO:45), or a conservatively substituted variant thereof. A conservatively substituted variant of a BH3-containing sequence means the sequence contains conservative amino acid substitutions of one or more of the amino acids in the naturally occurring sequence. The BH3 polypeptides of the invention can also include unusual amino acids and/or amino acids containing modifications such as glycosylations.

Preferred BH3 polypeptides are human BAX polypeptides BAX 53-76 (SEQ ID NO:31), BAX 57-71 (SEQ ID NO:33), BAX 61-71 (SEQ ID NO:35), and a human BID polypeptide, BID 81-100 (SEQ ID NO:37), which are defined by reference to the full-length BAX and BID sequences (FIGS. 21C and 21D). Most preferably, the BH3 polypeptide comprises human BAX 57-71 which consists of the sequence Lys-Lys-Leu-Ser-Glu-Cys-Leu-Lys-Arg-Ile-Gly-Asp-Glu-Leu-Asp (SEQ ID NO:33).

The invention also provides BH3 domain peptides having cell death agonist activity. A BH3 domain peptide

comprises five to eight contiguous amino acids from a BH3 domain as defined by SEQ ID NO:40, or a conservatively substituted variant thereof.

Methods for preparation of the BH3 polypeptides and BH3 domain peptides of the invention include, but are not limited to, chemical synthesis, recombinant DNA techniques or isolation from biological samples. Chemical synthesis of a peptide can be performed, for example, by the classical Merrifeld method of solid phase peptide synthesis (Merrifeld, *J Am Chem Soc 85:*2149, 1963 which is incorporated by reference) or the FMOC strategy on a Rapid Automated Multiple Peptide Synthesis system (DuPont Company, Wilmington, DE) (Caprino and Han, *J Org Chem* 37:3404, 1972 which is incorporated by reference).

The polypeptides and peptides of the present invention are also intended to include non-peptidal substances such as peptide mimetics which possess the death-inducing activity of BH3 polypeptides or BH3 domain peptides. The techniques for development of peptide mimetics are well known in the art. (See for example, Navia and Peattie, *Trends Pharm Sci 14*:189-195, 1993; Olson et al, *J Med Chem 36*:3039-3049 which are incorporated by reference). Typically this involves identification and characterization of the interaction between a protein target and its peptide ligand using X-ray crystallography and nuclear magnetic resonance technology. For example, it is believed that at least one target protein for BH3 polypeptides is the hydrophobic cleft formed by the BH1, BH2 and BH3 domains of an anti-apoptotic BCL-2 family member. Using information on a normal peptide-protein complex along with computerized molecular modeling, a pharmacophore hypothesis is developed and analogue compounds are made and tested in an assay system.

In one embodiment, the BH3 polypeptide or BH3 domain peptide is operably linked to a cell penetrating

agent. One such cell penetrating agent is the 11 amino acid Tat peptide of HIV-I (SEQ ID NO:55). The Tat peptide may be directly fused to the BH3 polypeptide or it may contain a short spacer sequence. The cell penetrating agent can also be a conservatively substituted variant of SEQ ID NO:55.

The present invention also includes therapeutic or pharmaceutical compositions comprising the BH3 polypeptide or BH3 domain peptide in an amount effective to promote death. Also encompassed within the present invention are methods for promoting apoptosis in a target cell comprising administering to the cell a death-promoting effective amount of the BH3 polypeptide. The target cell can be treated *ex vivo* or it can be present in a patient.

Such compositions and methods are useful for treating diseases or disease conditions in which the cell death signal is down regulated and the affected cell has an inappropriately diminished propensity for cell death, which is referenced herein as being a decreased apoptotic state. Such diseases include, for example, cancer, other lymphoproliferative conditions, arthritis, inflammation, autoimmune diseases and the like which may result from a down regulation of cell death regulation. The compositions and methods of the invention are also useful in treating diseases or disease conditions in which it is desirable to kill certain types of cells, such as virus-infected or autoantibody-expressing cells.

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including, for example, intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in *ex vivo* treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release

formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF). When it is intended that a BH3 polypeptide be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of the BH3 polypeptide across the blood-brain barrier.

The polypeptide can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, the BH3 polypeptide can be coupled to any substance known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor, and administered by intravenous injection. (See for example, Friden et al., *Science* 259:373-377, 1993 which is incorporated by reference). Furthermore, the BH3 polypeptide can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See for example Davis et al. *Enzyme Eng* 4:169-73, 1978; Burnham, *Am J Hosp Pharm* 51:210-218, 1994 which are incorporated by reference).

Furthermore, the compositions of the invention can also comprise agents which aid in targeting the BH3 polypeptide to a particular cell type and/or delivery into the cytosol of a cell. For example, the BH3 polypeptide can be encapsulated in liposomes that have various targeting ligands on their surface such as monoclonal antibodies that recognize antigens specifically expressed by the target cell or ligands which bind to receptors specific for the target cell. Such methods are well known in the art (see e.g., Amselem et al., *Chem Phys Lipids* 64:219-237, 1993 which is incorporated by reference). The BH3 polypeptide can also

be administered in a capsule comprised of a biocampatible polymer.

For nonparental administration, the compositions can also include absorption enhancers which increase the pore size of the mucosal membrane. Such absorption enhancers, which have been used to enable peptides the size of insulin to be transported across the mucosal membrane, include sodium deoxycholate, sodium glycocholate, dimethyl-β-cyclodextrin, lauroyl-1-lysophosphatidylcholine and other substances having structural similarities to the phospholipid domains of the mucosal membrane.

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. BID can also be incorporated into a solid or semi-solid biologically compatible matrix which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or

penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

It is also contemplated that certain formulations containing the BH3 polypeptide are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations

can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in cell death assays. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

In one embodiment of this invention, a BH3 polypeptide may be therapeutically administered by implanting into patients vectors or cells capable of producing a biologically-active form of the polypeptide or a precursor thereof, i.e. a molecule that can be readily converted to a biologically-active form of the BH3 polypeptide by the body. In one approach, cells transformed to express and secrete the BH3 polypeptide may be encapsulated into semipermeable membranes for implantation into a patient. It is preferred that the cell be of human origin and that the BH3 polypeptide have a human amino acid sequence when the patient is human. However, the formulations and methods herein can be used for veterinary as well as human applications and the term "patient" as used herein is intended to include human and veterinary patients.

Alternatively, the BH3 polypeptide can be administered to a target cell by transfecting the cell with a polynucleotide encoding for expression the BH3 polypeptide. If the target cell is in a patient the encoding polynucleotide can be targeted to the cell using methods known in the art, such as encapsulating the

polynucleotide in liposomes bearing targeting ligands or by non-covalently binding the polynucleotide to a ligand conjugate which directs the polynucleotide to the target cell. See, e.g., Wu et al., U.S. 5,635,383 and WO 95/25809.

The invention also provide polynucleotides encoding the BH3 polypeptides described herein. In particular, the polynucleotide comprises a nucleotide sequence encoding a BH3 domain consisting of the amino acid sequence set forth in SEQ ID NO:40. Preferred polynucleotides comprise a nucleotide sequence from one of the human cDNA sequences shown in Figure 22: bad (SEQ ID NO:47), bax (SEQ ID NO:48), bak (SEQ ID NO:49), bid (SEQ ID NO:50), or bik (SEQ ID NO:51).

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example 1

This example demonstrates that BAD contains a BH3 domain that is required for heterodimerization and cell death.

BAD was initially identified by its interaction with BCL-2 and BCL-X_L. To define the minimal region in BAD essential for its interaction with BCL-2, a nested set of deletion mutants was generated (Fig. 3A) and tested for their ability to interact with BCL-2 protein.

The deletion mutants were prepared by inserting fragments of a murine *bad* cDNA with engineered HindIII and EcoRI sites into the pET17b expression vector in

frame with the T7-gene-10 promoter and the resulting recombinant expression vectors were transformed into BL21 cells (Novagen). One hour after inducing expression of the truncated BAD proteins by IPTG (0.1 mM), total cell lysates were prepared. Lysates (40 µg) were size fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The resulting blot was hybridized with a ^{32}P-labeled glutathione s-transferase - BCL-2 (GST-BCL-2) fusion protein according to the protocol of Blanar and Rutter, *Science 256:*1014-1018, 1992, and the results are shown in Figure 2B.

Each of the BAD proteins 141-181, 141-172, 141-183, and 141-194 exhibited binding to GST-BCL-2 while the truncated BAD proteins 152-204, 163-204, and 173-204 did not bind to GST-BCL-2. Therefore, a small 31-amino acid region (BAD 141-172) is both sufficient and essential for BAD to heterodimerize with BCL-2.

Sequence analysis of this region identified a BAD amino acid sequence (151-159) with homology to BH3 domains found in other pro-apoptotic molecules (Fig. 4). The BH3 domain of BAD is predicted to be an amphipathic α-helix (Fig. 5).

Example 2

This example demonstrates that the BH3 domain is required for BAD's apoptotsis-promoting activity and that BAD deletion mutants lacking the BH3 domain do not bind to BCL-2 or BCL-X_L *in vitro*.

To assess the role of various regions of BAD in promoting apoptosis, full-length and various deletion mutants of BAD were transiently expressed in BAD-deficient murine embryonic fibroblasts (MEF). DNA fragments encoding for full-length BAD or truncated BAD proteins (1-181, 1-141, 127-204, and full-length with a deletion from 142 to 165) (Fig. 6A) and engineered to contain BamHI and EcoRI restriction sites were inserted

into pcDNA3 (Invitrogen), downstream of T7 and CMV promoters. MEF cells were allowed to grow to about 80% confluence in 12-well plates before transfection. A luciferase reporter plasmid (0.1 mg) was mixed with 0.05 mg of a pcDNA3 recombinant construct or the pcDNA3 vector as a control and 3 ml of lipofectAMINE™ (Gibco BRL) and 0.5 ml of the mixture was added to MEF cells for 5 hrs.

The transfected cells were lysed 18-20 hrs later and luciferase assays were performed using a standard substrate (Promega). Luciferase activities were quantified by a luminometer (OptocompII, MGM Instruments Inc.) and the relative luciferase activity for cells co-transfected with a recombinant pcDNA3 construct compared to luciferase activity in cells co-transfected with the control were determined. The means ± ISD of 3 experiments are shown in Fig. 6B.

The effect of recombinantly expressed full-length or truncated BAD on cell viability of the BAD-deficient MEF cells can be estimated by its effect on the activity of the co-transfected luciferase gene, with a low relative luciferase activity indicating low cell viability and high activity indicating good cell viability. As expected, lysates of cells co-transfected with full-length BAD (1-204) showed very little cell viability. In addition, two BAD truncated proteins, BAD 1-181, which was nearly full-length but lacked the BH2 domain, and BAD 127-204, which had a large N-terminal deletion but retained an intact BH1/BH3 region, were nearly as effective as full-length BAD in promoting cell death. In contrast, BAD constructs lacking the BH1/BH3 region (1-141 and Δ142-165) had substantially diminished death-promoting activity.

To assess the effect of this BH1/BH3 region on binding to anti-apoptotic members, an *in vitro* binding assay was performed. Equal amounts of *in vitro* translated, ^{35}S-labeled BCL-2 or BCL-X_L proteins were

incubated with 1 µg of purified GST-BAD fusion protein (wt or mutant) on ice for 30 min. 500 µl of NP-40 buffer with protease inhibitors and 25 µl of GSH-agarose was added to each binding mixture and rotated at 4°C for 1-2 hrs. Materials bound to GSH-agarose were precipitated, washed three times in 1 ml of NP-40 buffer, solubilized in 25 µl of 1X SDS-PAGE sample buffer, and electrophoresed on a 12.5% SDS polyacrylamide gel. An autoradiograph of the gel (not shown) showed that BAD full-length and deletion mutant constructs retaining the BH1/BH3 region formed heterodimers with BCL-2 and BCL-X_L, while BAD deletion mutants lacking the domain failed to bind BCL-2 or BCL-X_L (Fig. 6C). Thus, the BH1/BH3 region (142-165) is required for both heterodimerization and death agonist activity.

Example 3

This example demonstrates that binding of BAD to BCL-2 and BCL-X_L is affected by single amino acid changes in the BAD BH3 domain.

To further dissect the BH1/BH3 region of BAD, BAD mutant proteins were prepared with the following single-amino acid changes: Gly at position 148 to Ala (G148A); Arg at position 149 to Ala (R149A); and Leu at position 151 to Ala (BADL151A). These BAD mutants were generated by site-directed mutagenesis of a murine *bad* cDNA cloned into a pGEM-3Z derivative using the QuikChange site-directed mutagenesis kit (Stratagene). Sequence-confirmed mutant cDNAs and the wild-type murine *bad* cDNA were subcloned into the pSSFV expression vector. The resulting recombinants were used in an *in vitro* transcription-translation system (IVTT, Promega) to generate ^{35}S-labeled wild-type (WT) and mutant BAD proteins, which are shown in the upper panel of FIG. 7A (IVTT).

Binding of the ^{35}S-labeled wild-type and BH1/BH3 mutant BAD proteins to GST-BCL-2 and GST-BCL-X_L fusion proteins was assessed by an *in vitro* binding assay, which was performed as described in Example 2. The amount of radioactively labeled heterodimers captured on GSH agarose beads are shown in the middle and lower panels of FIG. 7A.

Substitutions in the region of BAD homologous to BH1 (G148A and R149A) did not significantly affect the ability of the BAD mutants to bind to BCL-X_L (FIG. 7A, lower panel). However, while binding to BCL-2 was not significantly affected by the R149A mutation, it was reduced approximately 50% by the G148A mutation (middle panel). Of note, replacement of Leu151 of the BH3 domain with alanine (L151A) reduced the binding of mutant BAD with either BCL-2 or BCL-X_L by more than 90%.

Example 4

This example demonstrates the ability of BAD BH1/BH3 mutants to bind to BCL-X_L *in vivo*.

The recombinant pSFFV expression vectors encoding the wild-type BAD and the BAD mutants described in Example 3 were electroporated into the murine hematopoietic cell line FL5.12 BCL-X_L, which overexpresses BCL-X_L. Clones expressing similar levels of WT and mutant BAD proteins as well as BCL-X_L were identified by probing Western blots of cell lysates with either a rabbit polyclonal anti-BAD antibody (#10929, described in Yang et al., *Cell 80:* 285-291, 1995) (Fig. 7B, upper panel) or a rabbit polyclonal anti-BCL-XL antibody (13.6, described in Boise et al., *Immunity 3:* 87-98, 1995) (Fig. 7B, lower panel).

To assess *in vivo* binding, BAD/BCL-X_L heterodimers were immunoprecipitated from cell lysates using 7B2, a murine monoclonal Ab against human BCL-X_L (Boise et al., *supra*). About 5-10 X 10^6 cells were lysed in 100 µl of

NP-40 isotonic lysis buffer with freshly added protease inhibitors (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.2], 1 mM EDTA, 0.25% NP-40, 0.2 mM PMSF, 0.1% aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin), incubated on ice for 30 min, and centrifuged at 15,000 X g for 10 min to precipitate nuclei and non-lysed cells. 20 µg of 7B2 mAb was added to the supernatant of each sample, mixed, and incubated on ice for 30 min. Subsequently 400 µl of NP-40 buffer was added to the sample along with 25 µl of protein A-sepharose and incubated at 4°C with rotation for 1-2 hrs. Immunoprecipitates were collected by a brief spin, washed three times with 1 ml of NP-40 buffer, and solubilized with 1X SDS-PAGE sample buffer. Total cell lysates, immunoprecipitated proteins and the remaining proteins in the BCL-X_L depleted samples were analyzed by western blot for the presence of BAD using the #10929 anti-BAD Ab. The results are shown in FIG. 7C, with the lane labeled IPα BCL-X_L representing the amount of BAD co-immunoprecipitated with BCL-X_L by the 7B2 mAb.

The mutants BAD G148A and BAD R149A were co-precipitated with BCL-X_L in amounts similar to that seen for wild-type BAD (FIG. 7C, compare lanes 2 and 5 with lane 11). However, 7B2 mAb co-precipitated greatly reduced amounts of BAD L151A with BCL-X_L as compared to wild-type BAD (FIG. 7C, compare lanes 8 and 11). Consistent with this, a markedly increased amount of BAD L151A was present in the supernatant (Sup) of this immunoprecipitate compared to the supernatants of the other mutants and wild-type (Sup, compare lane 9 with lanes 3, 6 and 12. This provides *in-vivo* confirmation of the *in vitro* binding results that the L151A mutation in the BH3 domain abolishes binding of BAD to BCL-X_L.

Example 5

This example demonstrates the effect of the BH1/BH3 mutations on intracellular distribution of BAD and apoptotic activity.

BAD is known to exist as a nonphosphorylated form that heterodimerizes with BCL-2 and BCL-X_L at membrane sites and as a hyperphosphorylated form that does not bind to BCL-2 or BCL-X_L but instead binds to the 14-3-3 protein in the cytosol (Zha et al., *supra*). To assess whether the loss of BCL-2 and BCL-X_L binding activity in the BAD L151A mutant corresponded with this intracellular distribution pattern, the inventors compared the intracellular distribution and 14-3-3 binding activity of wild-type BAD and the BH1/BH3 mutants.

The above-described FL5.12 cells co-expressing BCL-X_L and wild-type or mutant BAD proteins were washed with PBS twice, resuspended in Buffer A (10 mM Tris pH 7.5, 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, aprotinin 0.15 U/ml, 20 mM leupeptin, 1 mM PMSF) and incubated on ice for fifteen minutes. Cells were then homogenized in a Dounce homogenizer with fifty strokes and nuclei were removed by centrifugation at 500g for ten minutes. The supernatant was further centrifuged at 315,000g for thirty minutes to separate cytosol from crude membranes. Membrane fractions were solubilized in 1% SDS and centrifuged at 12,000g for five minutes at room temperature. The resulting membrane fractions and cytosol fractions were diluted 1:10 in 1% Triton X-100, 100 mM NaCl in buffer A and analyzed by western blot using the 10929 anti-BAD Ab and the results are shown in FIG. 8A.

The majority of BAD L151A was present in the cytosolic fraction (Cyt), with the more prominent upper band representing the hyperphosphorylated form and the lower band representing the nonphosphorylated form (Fig. 8A, lane 5). In contrast, the majority of wild-type BAD was detected as the nonphosphorylated form in the crude

membrane fraction (CM, lane 8) as was the majority of BAD G148A (lane 2). BAD R149A, which bears a mutation closer to the BH3 domain than G148A, displayed an intracellular distribution pattern that was intermediate between that observed for BAD G148A and L151A.

Binding ability to 14-3-3 was assessed by immunoprecipitation of BAD/14-3-3 complexes from the cytosolic fraction using the anti-BAD mAb 2G11 (Zha et al., *supra*). The amount of 14-3-3 protein in the immunoprecipitates was analyzed by western blot using an anti-14-3-3 antibody from Upstate Biotechnology, Inc., and the results are shown in FIG 8B.

The anti-BAD mAb 2G11 co-precipitated significantly more 14-3-3 protein associated with BAD L151A than with WT BAD or the other mutants. These data indicate that BAD L151A, which is incapable of binding to BCL-X_L, is also functionally inactive and localized to the cytosol where it is bound to 14-3-3.

Since FL5.12 BCL-XL cells expressing wild-type or mutant BAD are dependent upon IL-3 for survival, the viability of these cells was determined by propidium iodine exclusion at 24 hr., 48 hr., and 72 hr. after IL-3 withdrawal to assess the death-promoting ability of the BAD BH1/BH3 mutants. Two independent sets of clones selected for comparable levels of BAD expression were tested and showed similar results. The means ± ISD of triplicate assays are shown in FIG. 8C.

Like wild-type BAD, the mutants BAD G148A and BAD R149A, which have mutations within the BH1-like region, reversed the protective effect of BCL-X_L seen in the BCL-X_L/Hygro control. However, a high percentage of cells expressing BAD L151A were viable compared to the control, indicating this BH3 BAD mutant could no longer promote cell death.

Example 6

This example demonstrates that heterodimer formation between BAD and BCL-2 is destroyed by a single amino acid change in the BCL-2 BH3 domain.

To determine whether the BCL-2 BH3 domain played a role in BCL-2/BAD heterodimerization, three mutant BCL-2 proteins with single amino acid changes in the BH1, BH2 or BH3 domain, G145A, W188A, and L97A, respectively, were generated using site-directed mutagenesis and ^{35}S-labeled by IVTT essentially as described above. The location of the amino acid mutations are referenced with respect to the murine BCL-2 sequence of SEQ ID NO:?. The ability of the BCL-2 mutants to bind to a GST-wild-type BAD fusion protein (GST-BAD) was assessed in an *in vitro* binding assay performed as described above. As shown in FIG. 9, GST-BAD interacted with slightly reduced efficiency to the BCL-2 BH1 mutant (G145A) and weakly to the BH2 mutant (W188A), but not at all to the BCL-2 BH3 mutant (L97A). Thus, BH3 plays a prominent role in heterodimerization for both the death agonist and antagonist.

Example 7

This example illustrates the effect of BH3 domain mutations on the death agonist activity of BID and the binding of BID to BCL-2 or BAX.

The only conserved domain that BID possesses is BH3, prompting a mutational assessment of its functional importance (Figure 10A). BH3-mutant Bid constructs were generated in two steps. First, the 5' portion of the molecule was PCR amplified. The 5' primer added an *Eco*RI site, while the 3' primer ended at the *Nhe*I site 324 bp into the open reading frame. Second, the amplified *Eco*RI/*Nhe*I fragment plus the 3' *Nhe*I/*Eco*RI fragment were ligated into the *Eco*RI site of pBTM. Subsequently, the entire insert was subcloned into pSFFV for transfection into Fl5.12 cells, pcDNA3 for transient transfection,

pUHD10-3 for inducible clones in Jurkat cells and pGEX-HMK for GST-fusion proteins.

The BH3 mutants of BID were tested for their binding to BCL-2 and BAX *in vitro* (Figure 10B). All four mutants tested disrupted BID's interaction with either BCL-2 or BAX. However, the mutants did display different specificities: BIDmIII-1 (M97A,D98A) bound to BAX but not to BCL-2, BIDmIII-3 (G94A) bound to BCL-2 but not BAX, whereas BIDmIII-2 and mIII-4 did not bind to either (Figure 10B).

To determine if this *in vitro* binding data accurately reflected interactions of the BID mutants *in vivo*, we introduced each BID mutant into FL5.12-Bcl-2 cells and selected stable expressing clones. The expression level of BID mutants was comparable to that of a wild-type BID transfectant (Figure 11B). The ability of each mutant to interact with BCL-2 or BAX was assessed by immunoprecipitation with an anti-BID Ab followed by an anti-BCL-2 or anti-BAX immunoblot (Figure 11C). Anti-human-BCL-2 monoclonal Ab 6C8 and biotinylated anti-murine-BAX polyclonal Ab 651 were used for blot analyses (1:2000 and 1:500, respectively). Wild-type BID (lane 2) and BIDmIII-3 (lane 5) interacted with BCL-2 whereas wild-type BID and BIDmIII-1 (lane 3) interacted with BAX *in vivo*, confirming the *in vitro* binding data. BIDmIII-1 was the only mutant which still interacted with BAX, albeit a decreased amount similar to the *in vitro* assay (Figure 11C).

The capacity of BID mutants to counter protection by BCL-2 was assessed in the stably transfected FL5.12-Bcl-2 clones deprived of IL-3 (Figure 11A). Of note, all BH3 mutants of BID were impaired in their capacity to counter protection by BCL-2. Even BIDmIII-3 (G94A) which still avidly heterodimerized with BCL-2 was less effective than wild-type BID. This dissociated the capacity of BID to

form heterodimers with BCL-2 from its reversal of BCL-2 protection (Figure 10A).

This prompted further assessment of the BID mutants in the inducible system in Jurkat cells which does not require another apoptotic signal (Figure 12A). Moreover, Jurkat cells do not express substantial amounts of BCL-2. Despite substantial levels of protein (Figure 12B), BIDmIII-2,-3 & -4 displayed no meaningful death promoting effect (Figure 12A). Only BIDmIII-1 demonstrated substantial killing that was somewhat less than wt BID (Figure 12A), perhaps reflecting its weaker binding to BAX (Figures 10B and 11C). This BID mutant was also analyzed in the transient transfection death assay in Rat-1 fibroblasts. Once again, BIDmIII-1 demonstrated strong killing activity whereas, the activity of BIDmIII-3 & -4 was substantially impaired (Figure 12C). Thus, the BH3 mutations in BID score differently in stable transfectants with high levels of BCL-2 that require an external death stimulus (IL-3 deprivation, Figure 11A); when compared to systems which induce expression of BID and do not require another signal (Figures. 12A and 12C). Of note, the only BID mutant (mIII-1) still active (M97A,D98A) bound BAX but not BCL-2 (Figures 10B and 11C).

Site specific mutagenesis of BID revealed that BH3 was required for death promoting activity. This included the capacity to counter protection by BCL-2 as well as induce a cysteine protease dependent apoptosis when expressed in Jurkat T cells or Rat-1 fibroblasts (Table 1). The central glycine of BH3 was critical to BID's apoptotic activity.

Table 1

	BIDwt	BIDmIII-1	BIDmIII-2	BIDmIII-3	BIDmIII-4
Yeast Two-Hybrid Interactions with BCL-xL	+	-	-	+	-
In Vitro and *In Vivo* BCL-2 Binding	+	-	-	+	-
Counter BCL-2 *FL5.12-Bcl-2	+	-	-	-	-
In Vitro and *In Vivo* BAX Binding	+	+	-	-	-
Death Agonist Activity #Jurkat	+	+	-	-	-
•Rat-1	+	+	ND	-	-

* Ability to counteract BCL-2's death-inhibiting effect in FL5.12-Bcl=2 cells following IL-3 withdrawal;

Ability to induce cell death in Jurkat cells following induction of BID expression by Doxycyclin treatment;

• Transient co-transfection of both Bid and Luciferase plasmids into Rat-1 cells assessed by Luciferase assay.

Instructively, the various BH3 mutants of BID did not score identically in interactions with BCL-2 and BAX or in death agonist assays. BIDmIII-3 (G94A) which binds BCL-2 but not BAX lost its capacity to counter BCL-2 and induce apoptosis. In contrast, BIDmIII-1 (M97A,D98A) still bound BAX but not BCL-2 and retained death agonist activity. Furthermore, the failure of BIDmIII-1 to counter BCL-2 protection dissociates the capacity of BID to reverse BCL-2 protection from its binding to BCL-2. This provides evidence that BID restores apoptosis in

FL5.12-Bcl-2 cells by its death promoting activity that is independent of binding BCL-2 (Table 1).

Example 8

This example illustrates the effect of mutations in the BH3 domain on the dimerizing and death agonist activities of BAX.

Full-length BAX proteins with substitution mutations in or near the BH3 domain were prepared (Fig. 13A) and tested for their dimerization activity using a yeast two-hybrid binding assay. The following results were obtained: (1) all mutants except BAXmIII-1 (L63A, G67A, L70A, M74A) and BAXmIII-2 (L63E) retain the ability to interact with wild-type BAX, which suggests that in homodimers BH3 interacts with another domain(s), probably BH1 or BH2 or both; (2) BAXmIII-4 (G67E) and BAXmIII-5 (M74A) do not interact with BCL-2 and BCL-x_L; and (3) BAXmIII-3 (G67A), had no change in dimerization ability (Table 2).

Table 2

Summary of Bax Mutants in the BH3 Domain

	Yeast Two-Hybrid			*In Vivo* Interactions			Death Agonist Activity	Counteracting Bcl-2
	Bax	Bcl-2	Baxmut	Bax	Bcl-2	Baxmut		
Baxwt	+	+	NA	+	+	NA	+++	+++
mlll-1	-	-	-	-	-	-	+++	+++
mlll-2	-	-	-	-	-	-	+	-
mlll-3	+	+	+	+	+	+	+++	+
mlll-4	+	-	-	+	-	-	++	+
mlll-5	+	-	+	+	+	+	+++	+++

NA, not applicable

To reconfirm the binding specificity of BAX mutants *in vivo*, the polynucleotides encoding these mutants were subcloned into the mammalian expression vector pSFFV and introduced by electroporation into FL5.12 cells over-expressing BCL-2. Clones expressing exogenous HA-tagged mutant BAX were screened by Western blot with a polyclonal anti-BAX Ab 651, and those with the highest amount of expression were retained. Co-immunoprecipitations from ^{35}S-methionine labeled FL5.12-Bcl-2/HA-Bax cells with anti-HA and anti-BCL-2 antibodies confirmed most of the results by yeast two-hybrid system, with one exception: BAXmIII-5 binds to BCL-2 although it does not in yeast (data not shown). Thus the mutants were separated into three groups according to their binding specificity to BAX and BCL-2 in FL5.12 cells: BAXmIII-1 & 2, which do not bind to either; BAXmIII-4, which binds BAX but not BCL-2; and BAXmIII-3 & 5, which bind to both BAX and BCL-2 (Table 2).

To investigate the death-inducing activity of the BAX mutants, a transient transfection system in Rat-1 fibroblasts was used. BAX mutants were subcloned into the mammalian expression vector pcDNA3 under the control of a CMV promoter, and were co-transfected with a luciferase reporter into Rat-1 cells. Luciferase activity assays as described above were performed 16-18 hrs after transfection. Co-transfection of wild-type BAX with the luciferase reporter resulted in a 10-fold decrease in luciferase activity (Fig. 13B) reflecting its apoptosis activity. Mutants 1, 3 and 5 retained close to wild-type activity, while mutants 2 and 4 were 6- and 3-fold less potent then wild-type BAX, respectively (Fig. 13C).

To assess the ability of the BAX mutants to counteract the anit-apoptotic effect of BCL-2, the Rat-1 cells were co-transfected with polynucleotides encoding BCL-2 and wild-type BAX or a BAX mutant. As shown in FIG. 13C, co-transfection of wild-type BAX and BCL-2 resulted in an intermediate luciferase activity confirming the capacity of

BAX to counteract BCL-2. Mutants 1 and 5 retained wild-type like activity, mutant 2 lost 90% of the activity, while mutants 3 and 4 lost 50-60% of the activity.

The fact that BAXmIII-1 acted like wild-type in the functional assays was unexpected because it lost the ability to form dimers with wild-type BAX and BCL-2 based on the yeast two-hybrid and *in vivo* co-IP data. In order to know whether BAXmIII-1 could form homodimers, its ability for self-binding was tested with several assay systems. Results (data not shown) from yeast two-hybrid, *in vitro* binding and co-IP from transiently transfected 293 cells showed that while BAX mutants 3 and 5 form homodimers, BAX mutants 1, 2 and 4 almost completely lost their homodimerization activity.

A comparison of the interaction and cell killing activities of the BH3 mutants (Table 2) suggest that these two properties of BAX are separable. Moreover, the observation that BAXmIII-1 has no dimerizing activity but has death agonist activity suggests that the amphipathic character of the BH3 domain is sufficient for BAX to function as a death promoter.

Example 9

This example demonstrates the death-promoting activity of BAX and BID BH3-containing fragments when expressed in cells.

To assess the role of various regions of BAX and BID in promoting apoptosis, full-length and various deletion mutants (Figure 14A) were transiently expressed in Rat-1 cells with or without co-expression of BCL-2. DNA fragments encoding for full-length or truncated BAX and BAD proteins were engineered to contain BamHI and EcoRI restriction sites and inserted into pcDNA3 (Invitrogen) under the control of the CMV immediate early promoter. The recombinant pcDNA3 constructs, or the pcDN3 vector as a control, were lipo-transfected into Rat-1 cells along with a vector encoding a

luciferase reporter gene essentially as described in Example 2. In separate experiments, a recombinant pcDNA3 encoding BCL-2 was co-transfected. Luciferase activities were measured 20 hrs. after transfection as described above and expressed as the percentage of the control. The data are shown in FIG. 15A and 15B.

All BAX and BID fragments containing the BH3 domain displayed death agonist activity, as indicated by a reduction in luciferase activity compared to the control (FIG. 15A and 15B). Co-expression of BCL-2 countered the death agonist activity of these fragments. In contrast, cells expressing BID 1-73, which lacks the BH3 domain, were as viable as the control (vector, FIG. 15B).

The role of caspase activation in the cell death induced by BAX 53-104 and BID 74-128 was examined by culturing cells expressing these fragments or wild-type BAX or BID in the absence or presence of z-VAD-fmk (50 μM), which is a general caspase inhibitor (FIG. 15C). Although z-VAD-fmk did not significantly inhibit the death of cells expressing BAX wt but did significantly inhibit death of cells expressing BAX 53-104, BID wt, or BID 74-128.

The nuclear morphology of cells expressing BAX 53-104 or BID 74-128 was compared to that of cells expressing the respective full-length molecules by staining the cells with Hoechest 33342, which is a DNA-specific dye (Figure 16).

Example 10

This example demonstrates that small BH3-containing BAX and BID fragments fused to a tat-peptide can promote cell death.

Polypeptides containing an 11 amino acid sequence from the HIV-I Tat 1 protein (SEQ ID NO:48) and a wild-type or mutated BH3 domain (m) of BAX or BID with different lengths of flanking region (FIG. 17A) were chemically synthesized. The amino acid sequence in the mutated BH3 domains are scrambled versions of the sequential order of amino acids in

wild-type BH3 from BAX of BID. It is believed the Tat sequence facilitates entry of the polypeptide into the cells. These Tat-BH3 polypeptides were added to murine T cell hybridoma 2B4 cells at a concentration of 100 μM and cell viability was examined 4 hr. later by trypan blue dye exclusion.

As shown in Figure 17B, treatment of the 2B4 cells with Tat-BAX(53-76) (SEQ ID NO:31), Tat-BAX(57-71) (SEQ ID NO:33), Tat-Bax(61-71) (SEQ ID NO:35) and Tat-BID(81-100) (SEQ ID NO:37) fusion proteins resulted in a greater than 50% reduction in cell viability as determined by trypan blue dye exclusion at 4 hr. compared to viability in control cells with no treatment or treated with the Tat peptide. In contrast, the corresponding polypeptides containing mutated BH3 domains had no death agonist activity [Tat-BAX(53-76)M (SEQ ID NO:32), Tat-BAX(57-71)M (SEQ ID NO:34) and Tat-BID(81-100)M SEQ ID NO:38)]. The failure of Tat-BAX(53-86) and Tat-BID(75-106) to reduce cell viability in this assay is believed to be due to the larger size of these fusion polypeptides, which may inhibit their entry into the cells. Instructively, BAX53-86 displayed cell death agonist activity when expressed by cells (FIG. 15A) and Tat-BID(75-106) reduced viability of 2B4 cells by more than 40% when trypan blue dye exclusion was determined 19 hours after polypeptide addition (data not shown). This data suggests that therapeutic use of polypeptides longer than about 32 amino acids may require that they be administered with additional cell penetrating agents or expressed by polynucleotides transfected into the cell.

Example 11

This example demonstrates cell viability exposed illustrates the kinetics and dose-response relationship of cell death induced by Tat-BH3 polypeptides.

To assess longer term effects on cell death of the Tat-BH3 or Tat-BH3(m) fusion polypeptides, Tat-BAX(53-76), Tat-

BAX(67-71), Tat BID(81-100) or their corresponding BH3 mutant derivatives were added at a concentration of 100 μM to multiple sets of 2B4 cultures and trypan blue dye exclusion was determined at various times after polypeptide addition.

As shown in FIG. 18A, at concentrations of 100 μM, Tat-BID(81-100) achieved its maximum death promoting effect before the Tat-BAX fusion polypeptides, with more than 75% of the 2B4 cells losing viability by 1 hr. after addition of Tat-(BID)81-100 as compared to about 50% or 40% loss of viability in cells treated with Tat-BAX(57-71) or Tat-BAX(53-76), respectively. However, by 16 hours, the greatest reduction in cell viability was displayed by Tat-BAX(57-71), which killed almost all of the cells by that time, with about 15% and 35% of the cells treated with Tat-BID(81-100) and Tat-BAX(53-76) being viable. As expected, the mutant Tat-BH3 fusion polypeptides did not display significant cell killing activity at early times in the assay. Interestingly, one of these, Tat-BAX(57-71)m, reduced cell viability about 35% by 16 hours, indicating the mutant BH3 domain in this polypeptide has a low level of cell death agonist activity.

To assess the potency of these Tat-BH3 fusion polypeptides, Tat-BAX(57-71), Tat-BAX(57-71)m, Tat-BID(81-100), or Tat-BID(81-100)m was added to 2B4 cells at 25, 50, 75, 100, 125, or 150 μM and two hours later cell viability was determined by trypan blue dye exclusion. The results are shown in FIG. 18B.

The dose response curves for Tat-BAX(57-71) and Tat-BID(81-100) were similar, with loss of cell viability increasing with increasing doses of these polypeptides. While the polypeptides were about equally potent at 75 and 100 μM doses, Tat-BAX(57-71) killed a higher percentage of the 2B4 cells at 50 μM than a corresponding dose of Tat-BID(81-100). The Tat fusion polypeptides with mutant BH3

domains displayed no or very little effect on cell viability at all doses tested.

Example 12

This example illustrates that the cell death induced by Tat-BH3 fusion polypeptides is not inhibited by BCL-2 and z-VAD-fmk.

Duplicate cultures of 2B4 cells transfected with a recombinant vector encoding BCL-2 or control cells (neo) were treated with Tat-BAX(57-71) or Tat-BID(81-100) at 100 μM in the presence or absence of 100 μM of z-VAD-fmk. Two hours later, cell viability was measured by trypan blue dye exclusion (FIG. 19A) and the percentage of cells with subdiploid DNA (<2n) was determined by PI staining followed by flow cytometry (FIG. 19B).

In contrast to the cell death induced by BH3-containing fragments expressed in 2B4 cells, the cell death induced by Tat-BH3 polypeptides added to the cells in culture was not significantly reversed by BCL-2, z-VAD-fmk, or when both BCL-2 and z-VAD-fmk were present (FIG. 19A). Also, the percentage of cells with subdiploid DNA was significantly increased in cultures treated with one of the TatBH3 peptides and this increase was not significantly alleviated by z-VAD-fmk (FIG. 19B). Interestingly, the number of Tat-BID treated cells containing subdiploid DNA was reduced somewhat by BCL-2, but no significant reduction was seen for cells treated with Tat-BAX (FIG. 19B).

Example 13

This example demonstrates that cells treated with the Tat-BAX(57-71) or Tat(BID)81-100 polypeptides are morphologically atypical for apoptotic cells.

Jurkat cells were treated for 2 hours with 100 μM of Tat-BAX(57-71) (FIG. 20A, 20B) or Tat(BID)81-100 (FIG. 20C, 20D). The treated cells were stained with Hoechst 33342 and

then examined by phase contrast light microscopy (FIG. 20A, 20C) or fluorescent microscopy (FIG. 20B, 20D).

The light microscope study indicated that cells treated with these peptides had extensive cell membrane changes, including membrane blebbing. The nuclei of these cells, however, did not show the typical morphology seen in apoptosis in that they were not condensed nor fragmented. In most cases, the nuclei remained intact.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: WASHINGTON UNIVERSITY

(ii) TITLE OF INVENTION: CELL DEATH AGONISTS

(iii) NUMBER OF SEQUENCES: 55

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(E) COUNTRY: USA
(F) ZIP: 63105

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(C) REFERENCE/DOCKET NUMBER: 6029-6526

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(B) TELEFAX: 314-727-6092

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Arg Arg Met Ser Asp Glu Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Arg Arg Met Ser Asp Glu Phe Glu
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Ala Ile Ile Gly Asp Asp Ile Asn
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Ala Leu Ile Gly Asp Asp Ile Asn
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Arg Lys Ile Gly Asp Glu Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Arg Arg Ile Gly Asp Glu Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Ala Gln Val Gly Asp Ser Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Ala Gln Ile Gly Asp Glu Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Ala Cys Ile Gly Asp Glu Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Val Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Glu Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Gln Val Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Gln Val Gly Arg Gln Leu Ala Leu Ile Gly Asp Asp Ile Asn Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Lys Lys Leu Ser Glu Cys Leu Arg Lys Ile Gly Asp Glu Leu Asp Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Lys Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser Met Asp Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Ala Leu Ala Leu Arg Leu Ala Cys Ile Gly Asp Glu Met Asp Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Ala Gln Ile Gly Asp Glu Ala Ala His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Ala Gln Ala Ala Ala Ala Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Ala Gln Ile Ala Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Ala Gln Ile Glu Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Ser Glu Cys Ala Arg Arg Ile Ala Asp Glu Ala Asp Ser Asn Ala
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Leu Ser Glu Cys Glu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Leu Ser Glu Cys Leu Arg Arg Ile Ala Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Leu Ser Glu Cys Leu Arg Arg Ile Glu Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Ala
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp
1 5 10 15

Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile Ala Ala Val Asp
20 25 30

Thr Asp

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp
1 5 10 15

Glu Leu Asp Ser Asn Met Glu Leu
20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Glu Leu Asp Leu Lys Arg
1 5 10 15

Ile Gly Asp Ser Asn Met Glu Leu
20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp Glu Leu Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Lys Lys Leu Ser Glu Cys Glu Leu Asp Leu Lys Arg Ile Gly Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Glu Cys Leu Lys Arg Ile Gly Asp Glu Leu Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Asp Ser Glu Ser Gln Glu Glu Ile Ile His Asn Ile Ala Arg His Leu
1 5 10 15

Ala Gln Ile Gly Asp Glu Met Asp His Asn Ile Gln Pro Thr Leu Val
20 25 30

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Glu Ile Ile His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu
1 5 10 15

Met Asp His Asn
20

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Glu Ile Ile His Asn Ile Ala Arg His Gln Ile Gly Asp Glu Met Asp
1 5 10 15

Leu Ala His Asn
20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /note= "ARGININE OR ALANINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /note= "ARGININE, ISOLEUCINE, LEUCINE, LYSINE, GLUTAMIC ACID OR CYSTEINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /note= "METHIONINE, ISOLEUCINE OR VALINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "SERINE OR GLYCINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /note= "GLUTAMIC ACID, ASPARTIC ACID OR SERINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "PHENYLALANINE, ISOLEUCINE, LEUCINE OR METHIONINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "VALINE, GLUTAMIC ACID, ASPARAGINE OR ASPARTIC ACID"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Leu Xaa Xaa Xaa Xaa Asp Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 204 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Met Gly Thr Pro Lys Gln Pro Ser Leu Ala Pro Ala His Ala Leu Gly
1               5                  10                  15

Leu Arg Lys Ser Asp Pro Gly Ile Arg Ser Leu Gly Ser Asp Ala Gly
            20                  25                  30

Gly Arg Arg Trp Arg Pro Ala Ala Gln Ser Met Phe Gln Ile Pro Glu
        35                  40                  45

Phe Glu Pro Ser Glu Gln Glu Asp Ala Ser Ala Thr Asp Arg Gly Leu
    50                  55                  60

Gly Pro Ser Leu Thr Glu Asp Gln Pro Gly Pro Tyr Leu Ala Pro Gly
65                  70                  75                  80

Leu Leu Gly Ser Asn Ile His Gln Gln Gly Arg Ala Ala Thr Asn Ser
                85                  90                  95

His His Gly Gly Ala Gly Ala Met Glu Thr Arg Ser Arg His Ser Ser
            100                 105                 110

Tyr Pro Ala Gly Thr Glu Glu Asp Glu Gly Met Glu Glu Glu Leu Ser
        115                 120                 125

Pro Phe Arg Gly Arg Ser Arg Ser Ala Pro Pro Asn Leu Trp Ala Ala
    130                 135                 140

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Glu Gly
145                 150                 155                 160

Ser Phe Lys Gly Leu Pro Arg Pro Lys Ser Ala Gly Thr Ala Thr Gln
                165                 170                 175

Met Arg Gln Ser Ala Gly Trp Thr Arg Ile Ile Gln Ser Trp Trp Asp
            180                 185                 190

Arg Asn Leu Gly Lys Gly Gly Ser Thr Pro Ser Gln
        195                 200
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Gly Ala Gly Ala Val Glu Ile Arg Ser Arg His Ser Ser Tyr Pro Ala
1               5                  10                  15

Gly Thr Glu Asp Asp Glu Gly Met Gly Glu Glu Pro Ser Pro Phe Arg
            20                  25                  30
```

```
Gly Arg Ser Arg Ser Ala Pro Pro Asn Leu Trp Ala Ala Gln Arg Tyr
        35                  40                  45

Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Val Asp Ser Phe
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 208 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Lys Val Gly Cys Asp Glu
1               5                   10                  15

Ser Pro Ser Pro Ser Glu Gln Gln Val Ala Gln Asp Thr Glu Glu Val
            20                  25                  30

Phe Arg Ser Tyr Val Phe Tyr Leu His Gln Gln Glu Gln Glu Thr Gln
        35                  40                  45

Gly Arg Pro Pro Ala Asn Pro Glu Met Asp Asn Leu Pro Leu Glu Pro
    50                  55                  60

Asn Ser Ile Leu Gly Gln Val Gly Arg Gln Leu Ala Leu Ile Gly Asp
65                  70                  75                  80

Asp Ile Asn Arg Arg Tyr Asp Thr Glu Phe Gln Asn Leu Leu Glu Gln
                85                  90                  95

Leu Gln Pro Thr Ala Gly Asn Ala Tyr Glu Leu Phe Thr Lys Ile Ala
            100                 105                 110

Ser Ser Leu Phe Lys Ser Gly Ile Ser Trp Gly Arg Val Val Ala Leu
        115                 120                 125

Leu Gly Phe Gly Tyr Arg Leu Ala Leu Tyr Val Tyr Gln Arg Gly Leu
    130                 135                 140

Thr Gly Phe Leu Gly Gln Val Thr Cys Phe Leu Ala Asp Ile Ile Leu
145                 150                 155                 160

His His Tyr Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly Trp Val Ala
                165                 170                 175

Ala Leu Asn Leu Arg Arg Asp Pro Ile Leu Thr Val Met Val Ile Phe
            180                 185                 190

Gly Val Val Leu Leu Gly Gln Phe Val Val His Arg Phe Phe Arg Ser
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Arg Gln Glu Cys Gly Glu
1               5                  10                  15

Pro Ala Leu Pro Ser Ala Ser Glu Glu Gln Val Ala Gln Asp Thr Glu
            20                  25                  30

Glu Val Phe Arg Ser Tyr Val Phe Tyr Arg His Gln Gln Glu Gln Glu
        35                  40                  45

Ala Glu Gly Val Ala Ala Pro Ala Asp Pro Glu Met Val Thr Leu Pro
    50                  55                  60

Leu Gln Pro Ser Ser Thr Met Gly Gln Val Gly Arg Gln Leu Ala Ile
65                  70                  75                  80

Ile Gly Asp Asp Ile Asn Arg Arg Tyr Asp Ser Glu Phe Gln Thr Met
                85                  90                  95

Leu Gln His Leu Gln Pro Thr Ala Glu Asn Ala Tyr Glu Tyr Phe Thr
            100                 105                 110

Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly Ile Asn Trp Gly Arg Val
        115                 120                 125

Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu Ala Leu His Val Tyr Gln
    130                 135                 140

His Gly Leu Thr Gly Phe Leu Gly Gln Val Thr Arg Phe Val Val Asp
145                 150                 155                 160

Phe Met Leu His His Cys Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly
                165                 170                 175

Trp Val Ala Ala Leu Asn Leu Gly Asn Gly Pro Ile Leu Asn Val Leu
            180                 185                 190

Val Val Leu Gly Val Val Leu Leu Gly Gln Phe Val Val Arg Arg Phe
        195                 200                 205

Phe Lys Ser
    210
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Met Asp Gly Ser Gly Glu Gln Leu Gly Ser Gly Gly Pro Thr Ser Ser
1               5                   10                  15

Glu Gln Ile Met Lys Thr Gly Ala Phe Leu Leu Gln Gly Phe Ile Gln
            20                  25                  30

Asp Arg Ala Gly Arg Met Ala Gly Glu Thr Pro Glu Leu Thr Leu Glu
        35                  40                  45

Gln Pro Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Arg
    50                  55                  60

Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
65                  70                  75                  80

Ala Asp Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ala Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Val Trp Ile Gln Asp Gln Gly Gly Trp Glu Gly
145                 150                 155                 160

Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
Met Asp Gly Ser Gly Glu Gln Pro Arg Gly Gly Gly Pro Thr Ser Ser
1               5                   10                  15

Glu Gln Ile Met Lys Thr Gly Ala Leu Leu Leu Gln Gly Phe Ile Gln
            20                  25                  30

Asp Arg Ala Gly Arg Met Gly Gly Glu Ala Pro Glu Leu Ala Leu Asp
        35                  40                  45

Pro Val Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys
    50                  55                  60
```

```
Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
65                  70                  75                  80

Ala Ala Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ser Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Gly Trp Ile Gln Asp Gln Gly Gly Trp Asp Gly
145                 150                 155                 160

Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Met Asp Ser Glu Val Ser Asn Gly Ser Gly Leu Gly Ala Lys His Ile
1               5                   10                  15

Thr Asp Leu Leu Val Phe Gly Phe Leu Gln Ser Ser Gly Cys Thr Arg
            20                  25                  30

Gln Glu Leu Glu Val Leu Gly Arg Glu Leu Pro Val Gln Ala Tyr Trp
        35                  40                  45

Glu Ala Asp Leu Glu Asp Glu Leu Gln Thr Asp Gly Ser Gln Ala Ser
    50                  55                  60

Arg Ser Phe Asn Gln Gly Arg Ile Glu Pro Asp Ser Glu Ser Gln Glu
65                  70                  75                  80

Glu Ile Ile His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu
                85                  90                  95

Met Asp His Asn Ile Gln Pro Thr Leu Val Arg Gln Leu Ala Ala Gln
            100                 105                 110

Phe Met Asn Gly Ser Leu Ser Glu Glu Asp Lys Arg Asn Cys Leu Ala
        115                 120                 125

Lys Ala Leu Asp Glu Val Lys Thr Ala Phe Pro Arg Asp Met Glu Asn
    130                 135                 140
```

```
Asp Lys Ala Met Leu Ile Met Thr Met Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Ala Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Phe Ser Tyr Val Arg Asn Leu Val Arg Asn
            180                 185                 190

Glu Met Asp
        195
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
Met Asp Cys Glu Val Asn Asn Gly Ser Ser Leu Arg Asp Glu Cys Ile
1               5                   10                  15

Thr Asn Leu Leu Val Phe Gly Phe Leu Gln Ser Cys Ser Asp Asn Ser
            20                  25                  30

Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
        35                  40                  45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
    50                  55                  60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu
65                  70                  75                  80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
                85                  90                  95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
            100                 105                 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
        115                 120                 125

Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
    130                 135                 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
            180                 185                 190

Gly Met Asp
        195
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```
Met Ser Glu Val Arg Pro Leu Ser Arg Asp Ile Leu Met Glu Thr Leu
1               5                  10                  15

Leu Tyr Glu Gln Leu Leu Glu Pro Pro Thr Met Glu Val Leu Gly Met
            20                  25                  30

Thr Asp Ser Glu Glu Asp Leu Asp Pro Met Glu Asp Phe Asp Ser Leu
        35                  40                  45

Glu Cys Met Glu Gly Ser Asp Ala Leu Ala Leu Arg Leu Ala Cys Ile
    50                  55                  60

Gly Asp Glu Met Asp Val Ser Leu Arg Ala Pro Arg Leu Ala Gln Leu
65                  70                  75                  80

Ser Glu Val Ala Met His Ser Leu Gly Leu Ala Phe Ile Tyr Asp Gln
                85                  90                  95

Thr Glu Asp Ile Arg Asp Val Leu Arg Ser Phe Met Asp Gly Phe Thr
            100                 105                 110

Thr Leu Lys Glu Asn Ile Met Arg Phe Trp Arg Ser Pro Asn Pro Gly
        115                 120                 125

Ser Trp Val Ser Cys Glu Gln Val Leu Leu Ala Leu Leu Leu Leu Leu
    130                 135                 140

Ala Leu Leu Leu Pro Leu Leu Ser Gly Gly Leu His Leu Leu Leu Lys
145                 150                 155                 160
```

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 190 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
GGCGCTGGGG CTGTGGAGAT CCGGAGTCGC CACAGCTCCT ACCCCGCGGG GACGGAGGAC      60

GACGAAGGGA TGGGGGAGGA GCCCAGCCCC TTTCGGGGCC GCTCGCGCTC GGCGCCCCCC     120
```

AACCTCTGGG CAGCACAGCG CTATGGCCGC GAGCTCCGGA GGATGAGTGA CGAGTTTGTG 180

GACTCCTTTA 190

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2094 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GAGGATCTAC AGGGGACAAG TAAAGGCTAC ATCCAGATGC CGGGAATGCA CTGACGCCCA 60

TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC TGGGAGCAGC AGCCGCCAGC CCCTCGGACC 120

TCCATCTCCA CCCTGCTGAG CCACCCGGGT TGGGCCAGGA TCCCGGCAGG CTGATCCCGT 180

CCTCCACTGA GACCTGAAAA ATGGCTTCGG GGCAAGGCCC AGGTCCTCCC AGGCAGGAGT 240

GCGGAGAGCC TGCCCTGCCC TCTGCTTCTG AGGAGCAGGT AGCCCAGGAC ACAGAGGAGG 300

TTTTCCGCAG CTACGTTTTT TACCGCCATC AGCAGGAACA GGAGGCTGAA GGGGTGGCTG 360

CCCCTGCCGA CCCAGAGATG GTCACCTTAC CTCTGCAACC TAGCAGCACC ATGGGGCAGG 420

TGGGACGGCA GCTCGCCATC ATCGGGGACG ACATCAACCG ACGCTATGAC TCAGAGTTCC 480

AGACCATGTT GCAGCACCTG CAGCCCACGG CAGAGAATGC CTATGAGTAC TTCACCAAGA 540

TTGCCACCAG CCTGTTTGAG AGTGGCATCA ATTGGGGCCG TGTGGTGGCT CTTCTGGGCT 600

TCGGCTACCG TCTGGCCCTA CACGTCTACC AGCATGGCCT GACTGGCTTC CTAGGCCAGG 660

TGACCCGCTT CGTGGTCGAC TTCATGCTGC ATCACTGCAT TGCCCGGTGG ATTGCACAGA 720

GGGGTGGCTG GGTGGCAGCC CTGAACTTGG GCAATGGTCC CATCCTGAAC GTGCTGGTGG 780

TTCTGGGTGT GGTTCTGTTG GGCCAGTTTG TGGTACGAAG ATTCTTCAAA TCATGACTCC 840

CAAGGGTGCC CTTTGGGTCC CGGTTCAGAC CCCTGCCTGG ACTTAAGCGA AGTCTTTGCC 900

TTCTCTGTTC CCTTGCAGGG TCCCCCCTCA AGAGTACAGA AGCTTTAGCA AGTGTGCACT 960

CCAGCTTCGG AGGCCCTGCG TGGGGGCCAG TCAGGCTGCA GAGGCACCTC AACATTGCAT 1020

GGTGCTAGTG CCCTCTCTCT GGGCCCAGGG CTGTGGCCGT CTCCTCCCTC AGCTCTCTGG 1080

GACCTCCTTA GCCCTGTCTG CTAGGCGCTG GGGAGACTGA TAACTTGGGG AGGCAAGAGA 1140

CTGGGAGCCA CTTCTCCCCA GAAAGTGTTT AACGGTTTTA GCTTTTTATA ATACCCTTGT 1200

GAGAGCCCAT TCCCACCATT CTACCTGAGG CCAGGACGTC TGGGGTGTGG GGATTGGTGG 1260

GTCTATGTTC CCCAGGATTC AGCTATTCTG GAAGATCAGC ACCCTAAGAG ATGGGACTAG 1320

GACCTGAGCC TGGTCCTGGC CGTCCCTAAG CATGTGTCCC AGGAGCAGGA CCTACTAGGA 1380

GAGGGGGGCC AAGGTCCTGC TCAACTCTAC CCCTGCTCCC ATTCCTCCCT CCGGCCATAC 1440

TGCCTTTGCA GTTGGACTCT CAGGGATTCT GGGCTTGGGG TGTGGGGTGG GGTGGAGTCG 1500

CAGACCAGAG CTGTCTGAAC TCACGTGTCA GAAGCCTCCA AGCCTGCCTC CCAAGGTCCT 1560

CTCAGTTCTC TCCCTTCCTC TCTCCTTATA GACACTTGCT CCCAACCCAT TCACTACAGG 1620

TGAAGGCTCT CACCCATCCC TGGGGGCCTT GGGTGAGTGG CCTGCTAAGG CTCCTCCTTG 1680

CCCAGACTAC AGGGCTTAGG ACTTGGTTTG TTATATCAGG GAAAAGGAGT AGGGAGTTCA 1740

TCTGGAGGGT TCTAAGTGGG AGAAGGACTA TCAACACCAC TAGGAATCCC AGAGGTGGAT 1800

CCTCCCTCAT GGCTCTGGCA CAGTGTAATC CAGGGGTGTA GATGGGGGAA CTGTGAATAC 1860

TTGAACTCTG TTCCCCCACC CTCCATGCTC CTCACCTGTC TAGGTCTCCT CAGGGTGGGG 1920

GGTGACAGTG CCTTCTCTAT TGGCACAGCC TAGGGTCTTG GGGGTCAGGG GGGAGAAGTT 1980

CTTGATTCAG CCAAATGCAG GGAGGGGAGG CAGATGGAGC CCATAGGCCA CCCCCTATCC 2040

TCTGAGTGTT TGGAAATAAA CTGTGCAATC CCCTCAAAAA AAAAACGGAG ATCC 2094

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 579 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

ATGGACGGGT CCGGGGAGCA GCCCAGAGGC GGGGGGCCCA CCAGCTCTGA GCAGATCATG 60

AAGACAGGGG CCCTTTTGCT TCAGGGTTTC ATCCAGGATC GAGCAGGGCG AATGGGGGGG 120

GAGGCACCCG AGCTGGCCCT GGACCCGGTG CCTCAGGATG CGTCCACCAA GAAGCTGAGC 180

GAGTGTCTCA AGCGCATCGG GGACGAACTG GACAGTAACA TGGAGCTGCA GAGGATGATT 240

GCCGCCGTGG ACACAGACTC CCCCCGAGAG GTCTTTTTCC GAGTGGCAGC TGACATGTTT 300

TCTGACGGCA ACTTCAACTG GGGCCGGGTT GTCGCCCTTT TCTACTTTGC CAGCAAACTG 360

GTGCTCAAGG CCCTGTGCAC CAAGGTGCCG GAACTGATCA GAACCATCAT GGGCTGGACA 420

TTGGACTTCC TCCGGGAGCG GCTGTTGGGC TGGATCCAAG ACCAGGGTGG TTGGGACGGC 480

CTCCTCTCCT ACTTTGGGAC GCCCACGTGG CAGACCGTGA CCATCTTTGT GGCGGGAGTG 540

CTCACCGCCT CGCTCACCAT CTGGAAGAAG ATGGGCTGA 579

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 588 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

ATGGACTGTG AGGTCAACAA CGGTTCCAGC CTCAGGGATG AGTGCATCAC AAACCTACTG 60

GTGTTTGGCT TCCTCCAAAG CTGTTCTGAC AACAGCTTCC GCAGAGAGCT GGACGCACTG 120

GGCCACGAGC TGCCAGTGCT GGCTCCCCAG TGGGAGGGCT ACGATGAGCT GCAGACTGAT 180

GGCAACCGCA GCAGCCACTC CCGCTTGGGA AGAATAGAGG CAGATTCTGA AAGTCAAGAA 240

GACATCATCC GGAATATTGC CAGGCACCTC GCCCAGGTCG GGGACAGCAT GGACCGTAGC 300

ATCCCTCCGG GCCTGGTGAA CGGCCTGGCC CTGCAGCTCA GGAACACCAG CCGGTCGGAG 360

GAGGACCGGA ACAGGGACCT GGCCACTGCC CTGGAGCAGC TGCTGCAGGC CTACCCTAGA 420

GACATGGAGA AGGAGAAGAC CATGCTGGTG CTGGCCCTGC TGCTGGCCAA GAAGGTGGCC 480

AGTCACACGC CGTCCTTGGC TCCGTGATGT CTTTCACACA ACAGTAATTT TATTAACCAG 540

AACCTACGCA CCTACGTGAG GAGCTTAGCC AGAAATGGGA TGGACTGA 588

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 923 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CAGCATCGCC GCCGCCAGAG GAGAAATGTC TGAAGTAAGA CCCCTCTCCA GAGACATCTT 60

GATGGAGACC CTCCTGTATG AGCAGCTCCT GGAACCCCCG ACCATGGAGG TTCTTGGCAT 120

GACTGACTCT GAAGAGGACC TGGACCCTAT GGAGGACTTC GATTCTTTGG AATGCATGGA 180

GGGCAGTGAC GCATTGGCCC TGCGGCTGGC CTGCATCGGG GACGAGATGG ACGTGAGCCT 240

CAGGGCCCCG CGCCTGGCCC AGCTCTCCGA GGTGGCCATG CACAGCCTGG GTCTGGCTTT 300

CATCTACGAC CAGACTGAGG ACATCAGGGA TGTTCTTAGA AGTTTCATGG ACGGTTTCAC 360

CACACTTAAG GAGAACATAA TGAGGTTCTG GAGATCCCCG AACCCCGGGT CCTGGGTGTC 420

CTGCGAACAG GTGCTGCTGG CGCTGCTGCT GCTGCTGGCG CTGCTGCTGC CGCTGCTCAG 480

CGGGGGCCTG CACCTGCTGC TCAAGTGAGC CCCCGGCGGC TCAGGCGTGG CTGGCCCCAC 540

CCCCATGACC ACTGCCCTGA GGTGGCGGCC TGCTGCTGTT ATCTTTTTAA CTGTTTTCTC 600

ATGATGCCTT TTATATTAAC CCCGTGATAG TGCTGGAACA CTGCTGAGGT TTTATACTCA 660

GGTTTTTTGT TTTTTTTTTA TTCCAGTTTT CGTTTTTTCT AAAAGATGAA TTCCTATGGC 720

TCTGCAATTG TCACCGGTTA ACTGTGGCCT GTGCCCAGGA AGAGCCATTC ACTCCTGCCC 780

CTGCCCACAC GGCAGGTAGC AGGGGGAGTG CTGGTCACAC CCCTGTGTGA TATGTGATGC 840

CCTCGGCAAA GAATCTACTG GAATAGATTC CGAGGAGCAG GAGTGCTCAA TAAAATGTTG 900

GTTTCCAGCA AAAAAAAAAA AAA 923

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg
1 5 10

What is Claimed is:

1. A bcl-homology domain 3 polypeptide (BH3 polypeptide) comprising a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) the BH3 polypeptide consists of no more than 50 contiguous amino acids, and

(c) the BH3 polypeptide has cell death agonist activity.

2. The BH3 polypeptide of claim 1, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a conservative substituted variant thereof.

3. The BH3 polypeptide of claim 1, which comprises 15 to 24 contiguous amino acids.

4. The BH3 polypeptide of claim 1, which comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

5. The BH3 polypeptide of claim 1, which comprises a human BID polypeptide consisting of SEQ ID NO:37.

6. The BH3 polypeptide of claim 1 which is operably linked to a cell penetrating agent.

7. The BH3 polypeptide of claim 7, wherein the cell-penetrating agent is a Tat peptide as set forth in SEQ ID NO:55 or a conservatively substituted thereof.

8. A polynucleotide encoding a BH3 polypeptide which comprises a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) the BH3 polypeptide consists of no more than 50 contiguous amino acids, and

(c) the BH3 polypeptide has cell death agonist activity.

9. The polynucleotide of claim 8, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a conservative substituted variant thereof.

10. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises 15 to 24 contiguous amino acids.

11. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

12. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises a human BID polypeptide consisting of SEQ ID NO:37.

13. A method for promoting apoptosis in a target cell comprising administering to the cell a death-promoting effective amount of a BH3 polypeptide which comprises a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) consists of no more than 50 contiguous amino acids, and

(c) has cell death agonist activity.

14. The method of claim 13, wherein the target cell is present in a human patient and is a cancer cell, a virus-infected cell, or an auto-antibody-producing cell.

15. The method of claim 14, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

16. The method of claim 14, wherein the BH3 polypeptide comprises 15 to 24 contiguous amino acids.

17. The method of claim 14, wherein the BH3 polypeptide comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

18. The method of claim 14, wherein the BH3 polypeptide comprises a human BID fragment consisting of SEQ ID NO:37.

19. The method of claim 14, wherein the BH3 polypeptide is operably linked to a cell penetrating agent.

20. The method of claim 14, wherein the administering step comprises transfecting the cell with a polynucleotide encoding for expression the BH3 polypeptide.

21. A bcl-homology domain 3 peptide (BH3 domain peptide) comprising five to eight amino acids from a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family, and

(b) the BH3 domain peptide has cell death agonist activity.

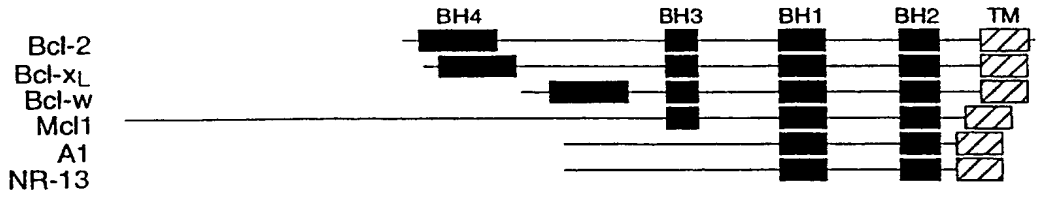
FIGURE 1

hBAD		L	R	R	M	S	D	E	F	V			SEQ ID NO:1
mBAD	151	L	R	R	M	S	D	E	F	E	159		SEQ ID NO:2
hBAK	78	L	A	I	I	G	D	D	I	N	86		SEQ ID NO:3
mBAK	75	L	A	L	I	G	D	D	I	N	83		SEQ ID NO:4
hBAX	63	L	R	K	I	G	D	E	L	D	71		SEQ ID NO:5
mBAX	63	L	R	R	I	G	D	E	L	D	71		SEQ ID NO:6
hBID	90	L	A	Q	V	G	D	S	M	D	98		SEQ ID NO:7
mBID	90	L	A	Q	I	G	D	E	M	D	98		SEQ ID NO:8
hBIK	61	L	A	C	I	G	D	E	M	D	69		SEQ ID NO:9

THE BCL-2 FAMILY

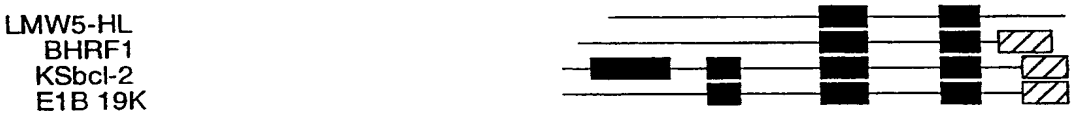
ANTI-APOPTOTIC

MAMMALIAN

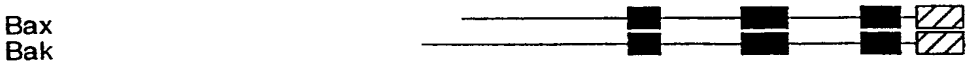


C. elegans

VIRAL HOMOLOGS



PRO-APOPTOTIC



PRO-APOPTOTIC — BH3

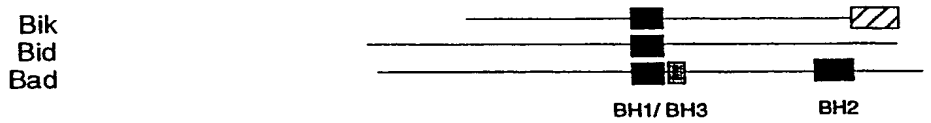


FIGURE 2

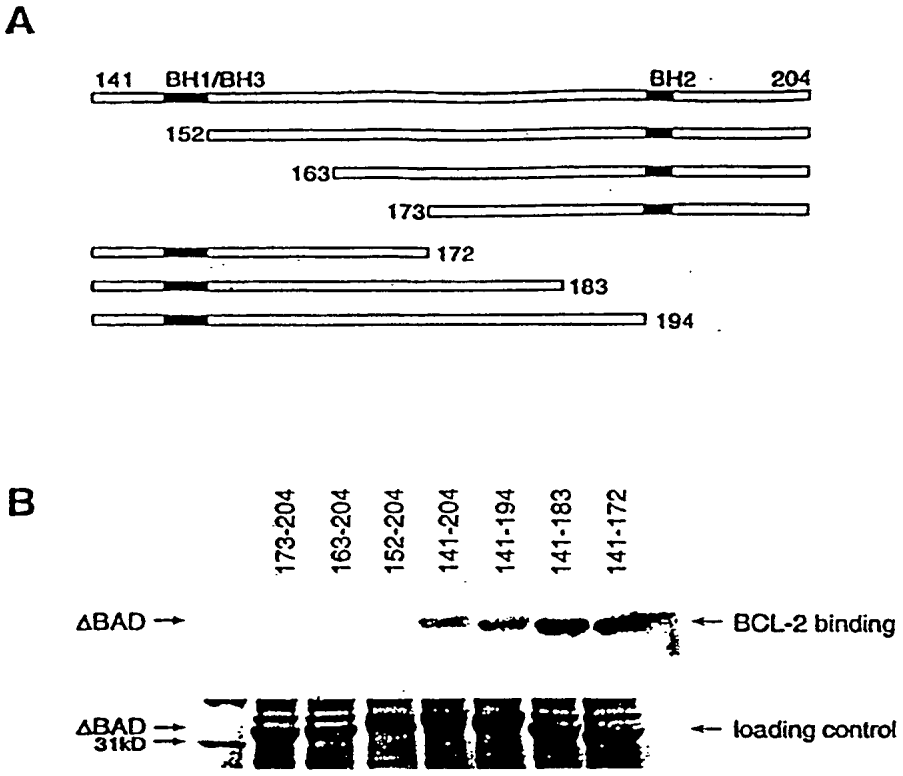

A
141
BH1/BH3
BH2
204
152
163
173
172
183
194
B
173-204
163-204
152-204
141-204
141-194
141-183
141-172
ΔBAD
BCL-2 binding
ΔBAD
31kD
loading control

Figure 3

Figure 4

hBAD		Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	V	D		SEQ ID NO:10
mBAD	145	Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	E	G	160	SEQ ID NO:11
Hbak	72	G	Q	V	G	R	Q	L	A	I	I	G	D	D	I	N	R	87	SEQ ID NO:12
mBAK	69	G	Q	V	G	R	Q	L	A	L	I	G	D	D	I	N	R	84	SEQ ID NO:13
hBAX	57	K	K	L	S	E	C	L	R	K	I	G	D	E	L	D	S	72	SEQ ID NO:14
mBAX	57	K	K	L	S	E	C	L	R	R	I	G	D	E	L	D	S	72	SEQ ID NO:15
hBID	84	R	N	I	A	R	H	L	A	Q	V	G	D	S	M	D	R	99	SEQ ID NO:16
mBID	84	H	N	I	A	R	H	L	A	Q	I	G	D	E	M	D	H	99	SEQ ID NO:17
hBIK	55	D	A	L	A	L	R	L	A	C	I	G	D	E	M	D	V	70	SEQ ID NO:18

BH3 Domain

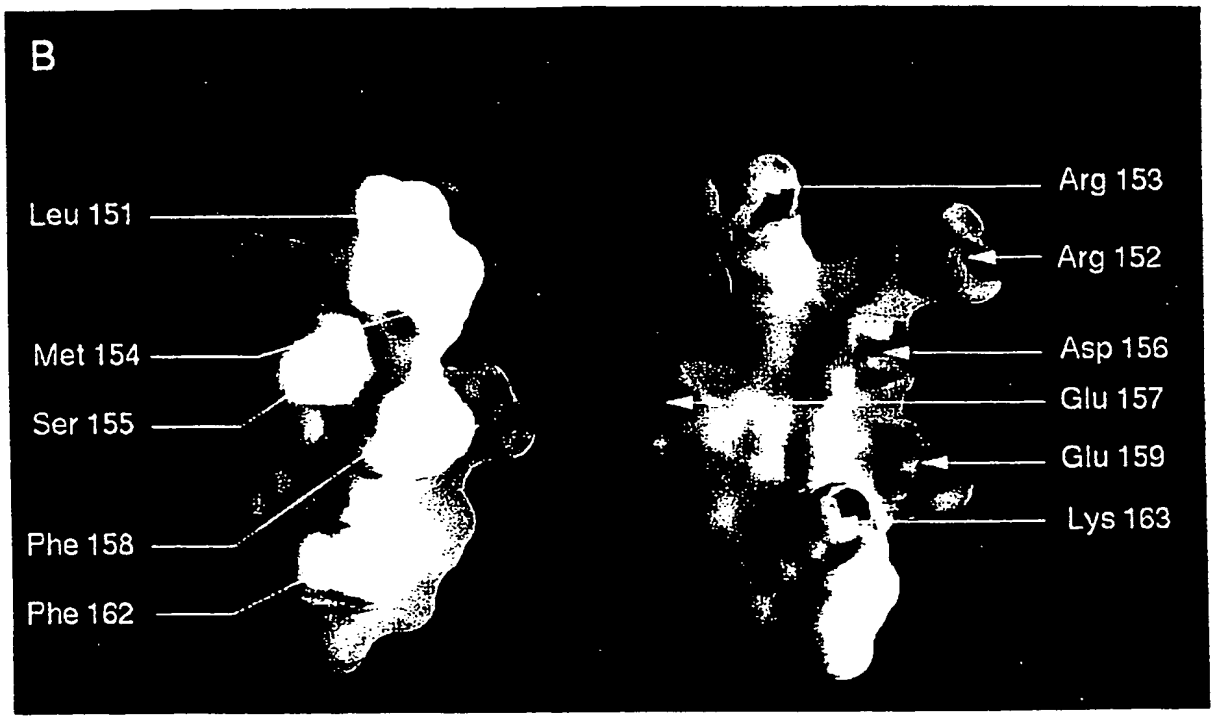


FIGURE 5

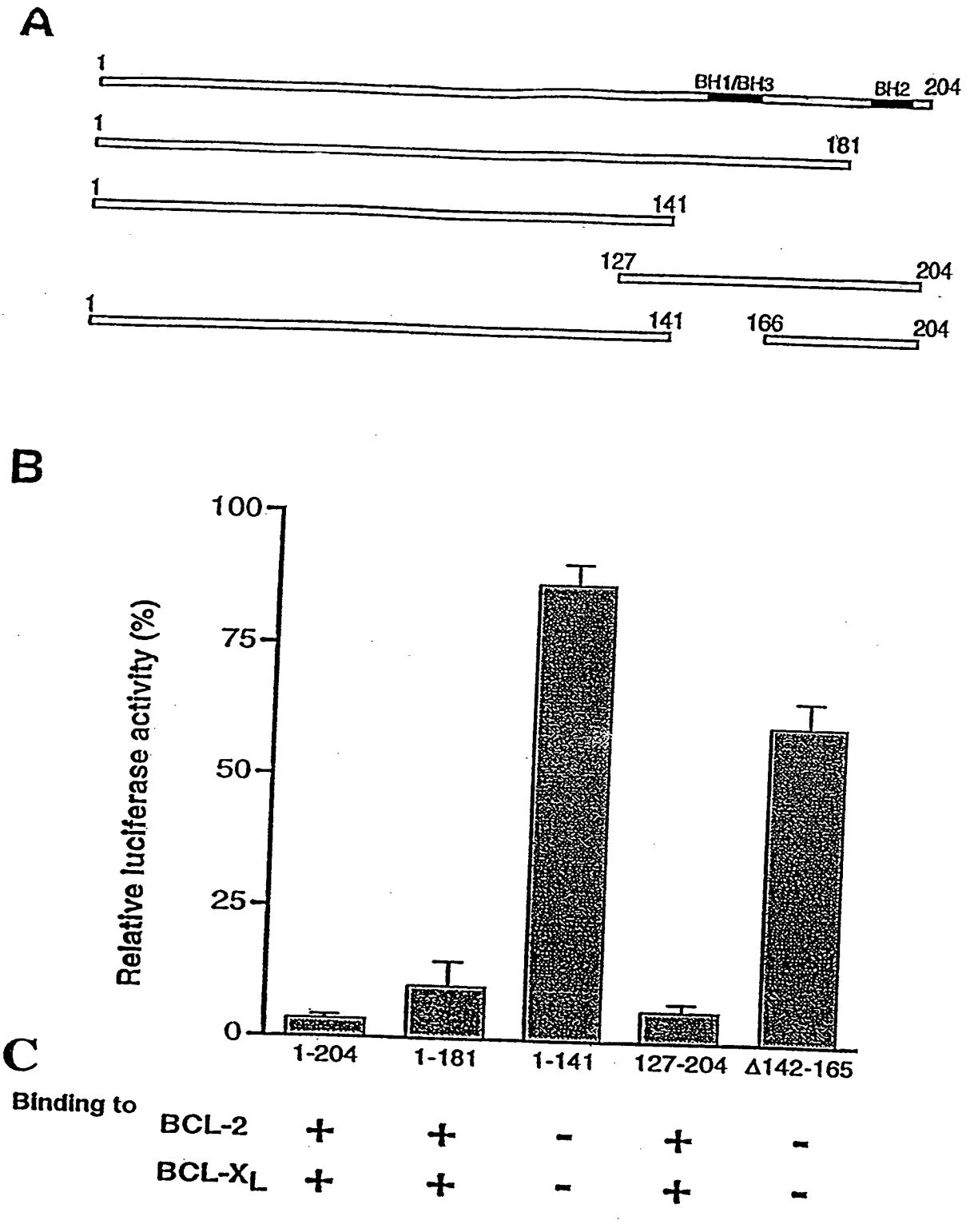

A
1
BH1/BH3
BH2
204
1
181
1
141
127
204
1
141
166
204
B
Relative luciferase activity (%)
100
75
50
25
0
1-204
1-181
1-141
127-204
Δ142-165
C
Binding to
BCL-2 + + - + -
BCL-XL + + - + -

Figure 6

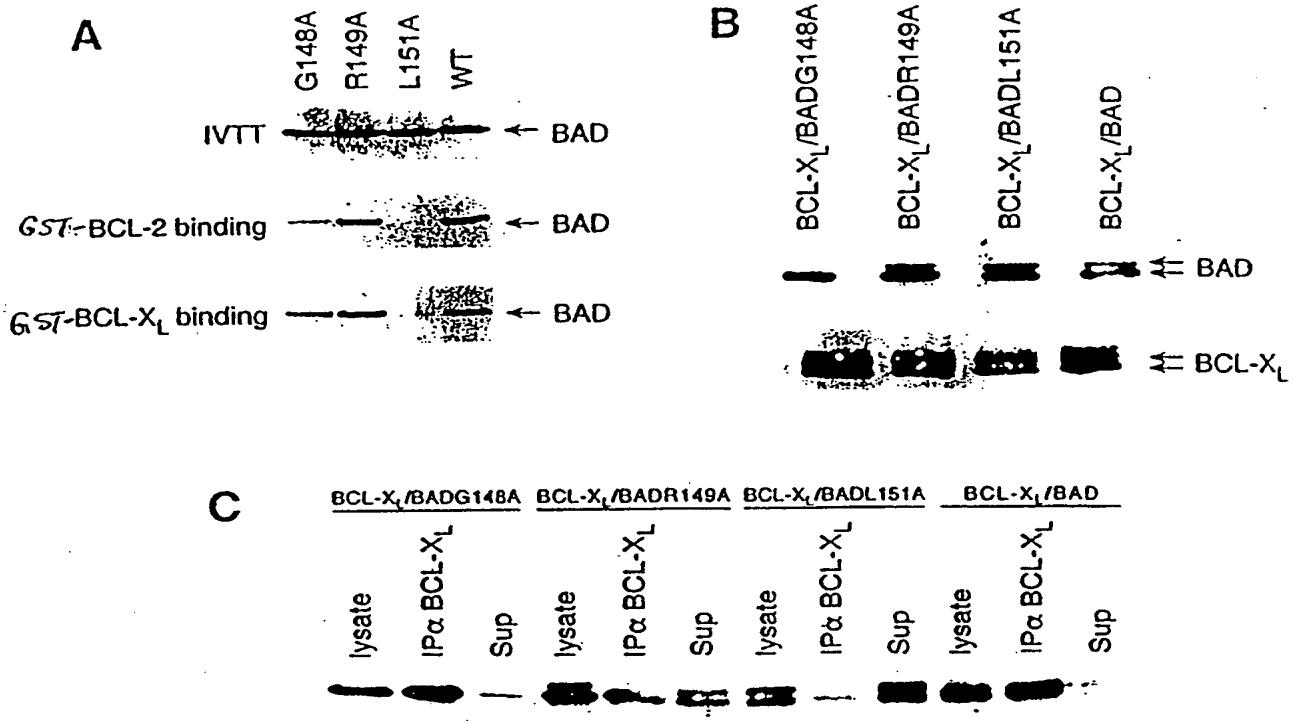


Figure 7

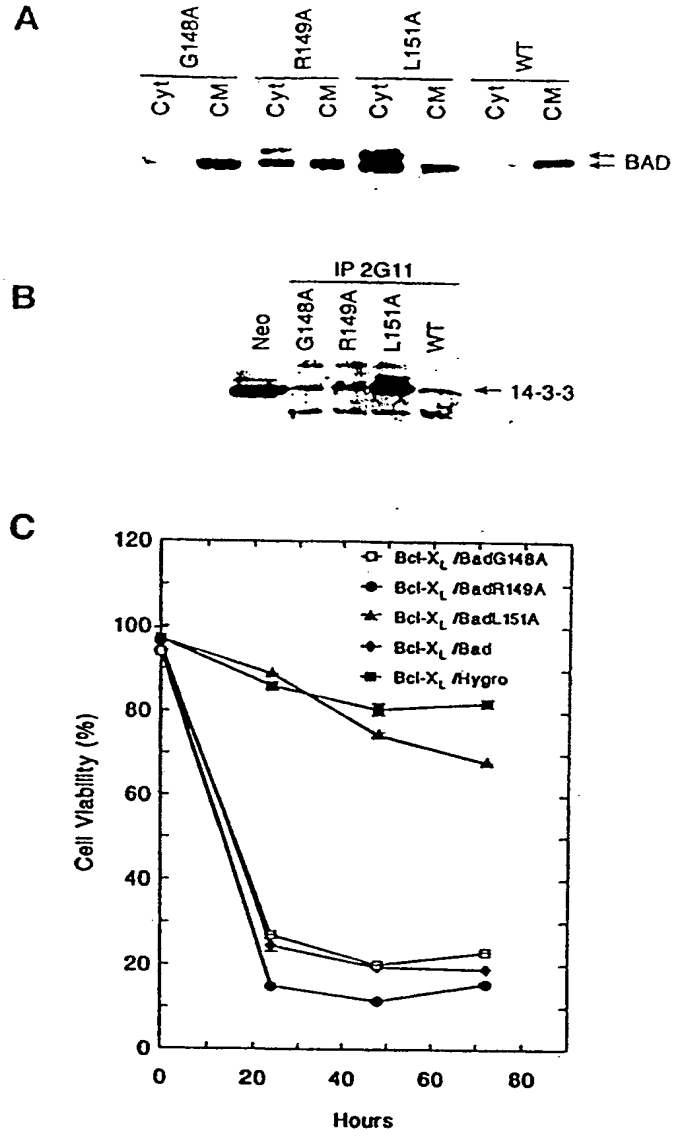

A
G148A
R149A
L151A
WT
Cyt
CM
BAD
B
IP 2G11
Neo
G148A
R149A
L151A
WT
14-3-3
C
Bcl-X$_L$ /BadG148A
Bcl-X$_L$ /BadR149A
Bcl-X$_L$ /BadL151A
Bcl-X$_L$ /Bad
Bcl-X$_L$ /Hygro
Cell Viability (%)
Hours
0
20
40
60
80
100
120

Figure 8

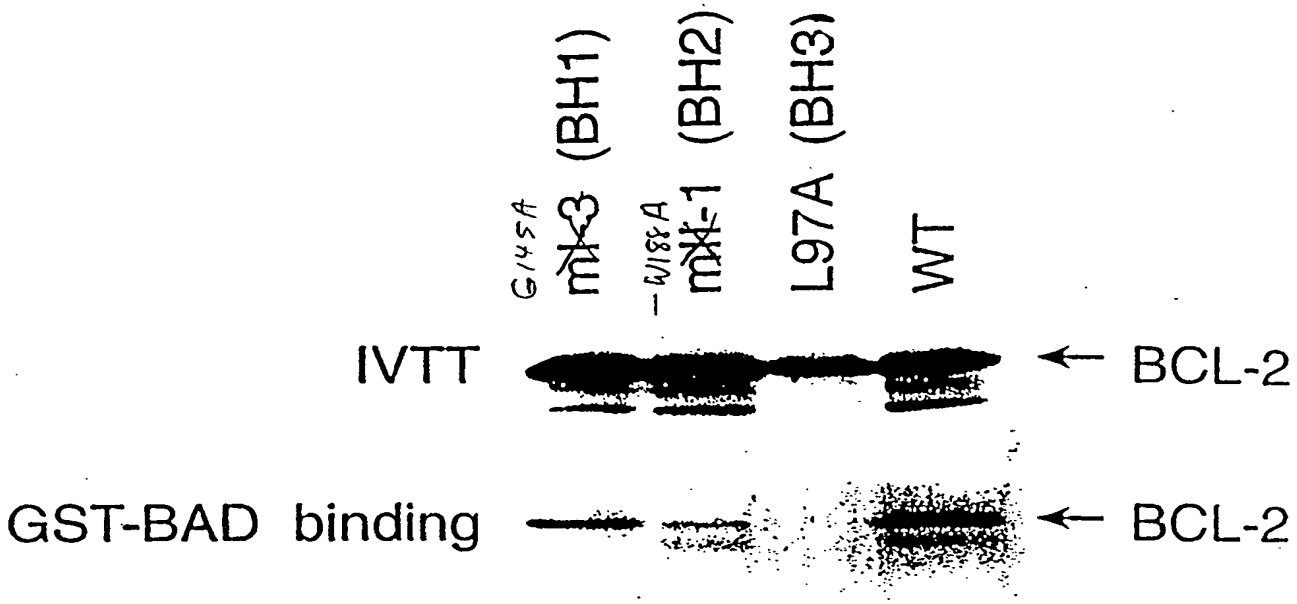

G145A
Mx-3 (BH1)
-W181A
Mx-1 (BH2)
L97A (BH3)
WT
IVTT
BCL-2
GST-BAD binding
BCL-2

Figure 9

Figure 10A

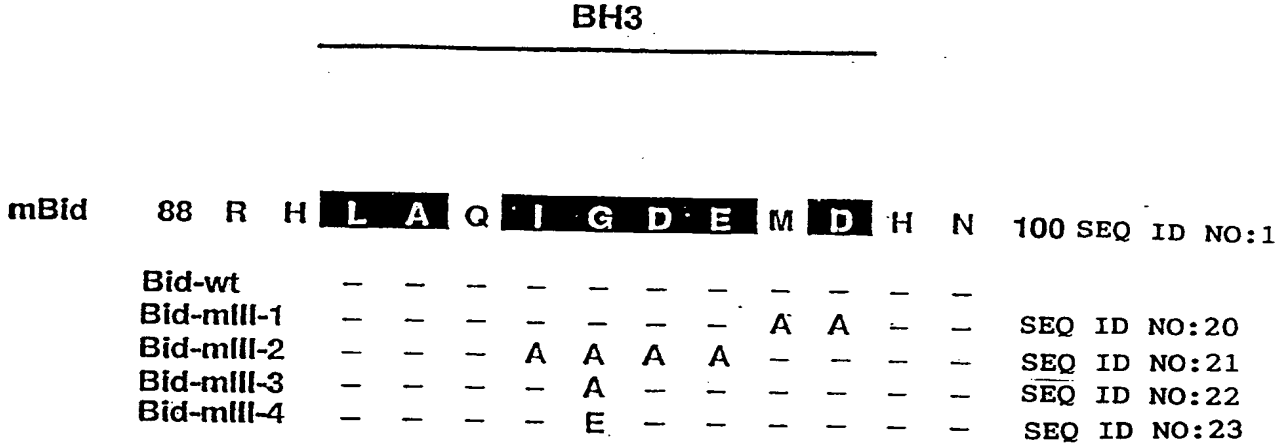


Figure 10B

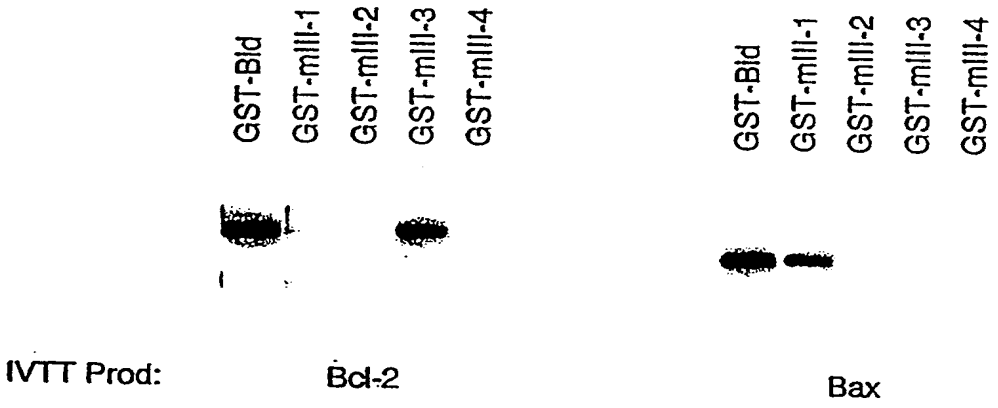


Figure 11A

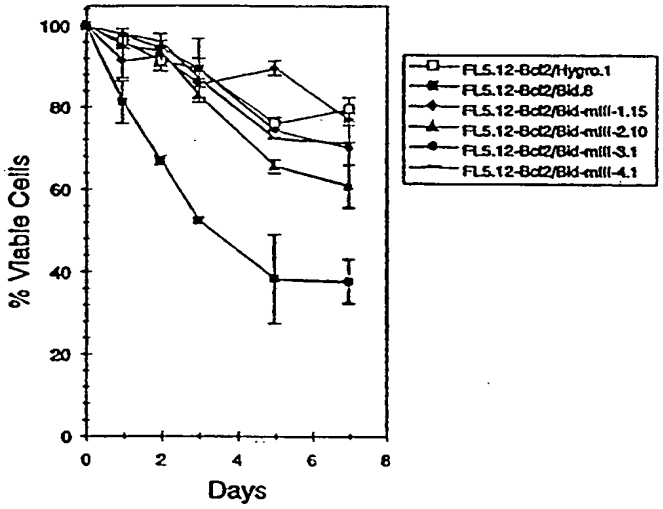


Figure 11B

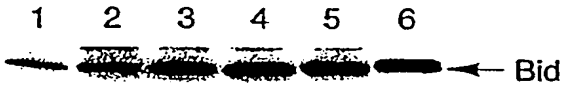


Figure 11C

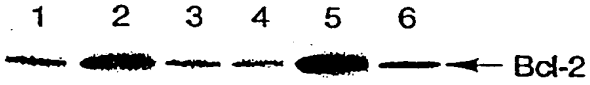


Figure 12B

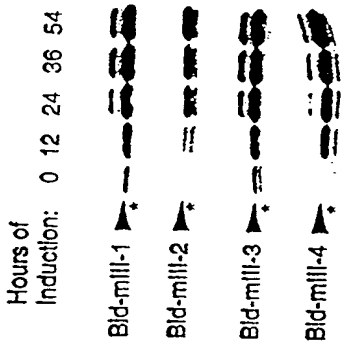


Figure 12A

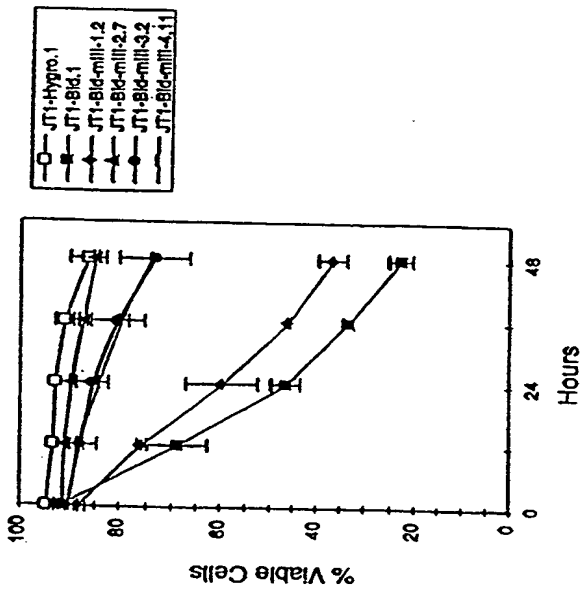
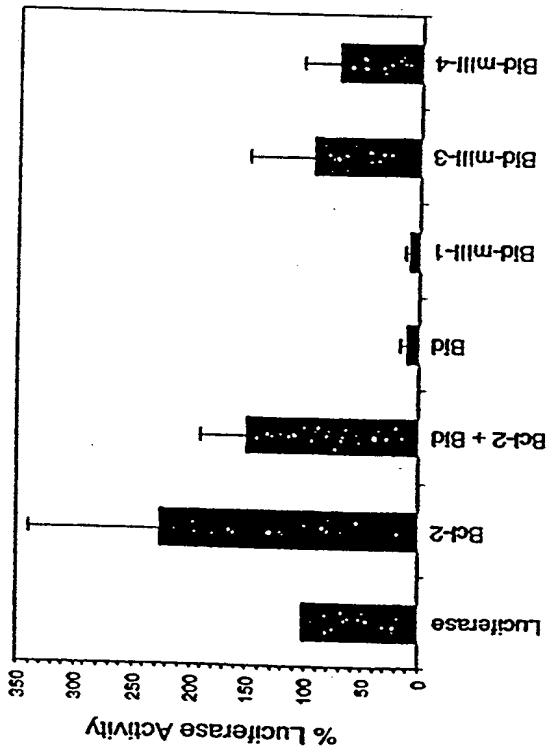


Figure 12C



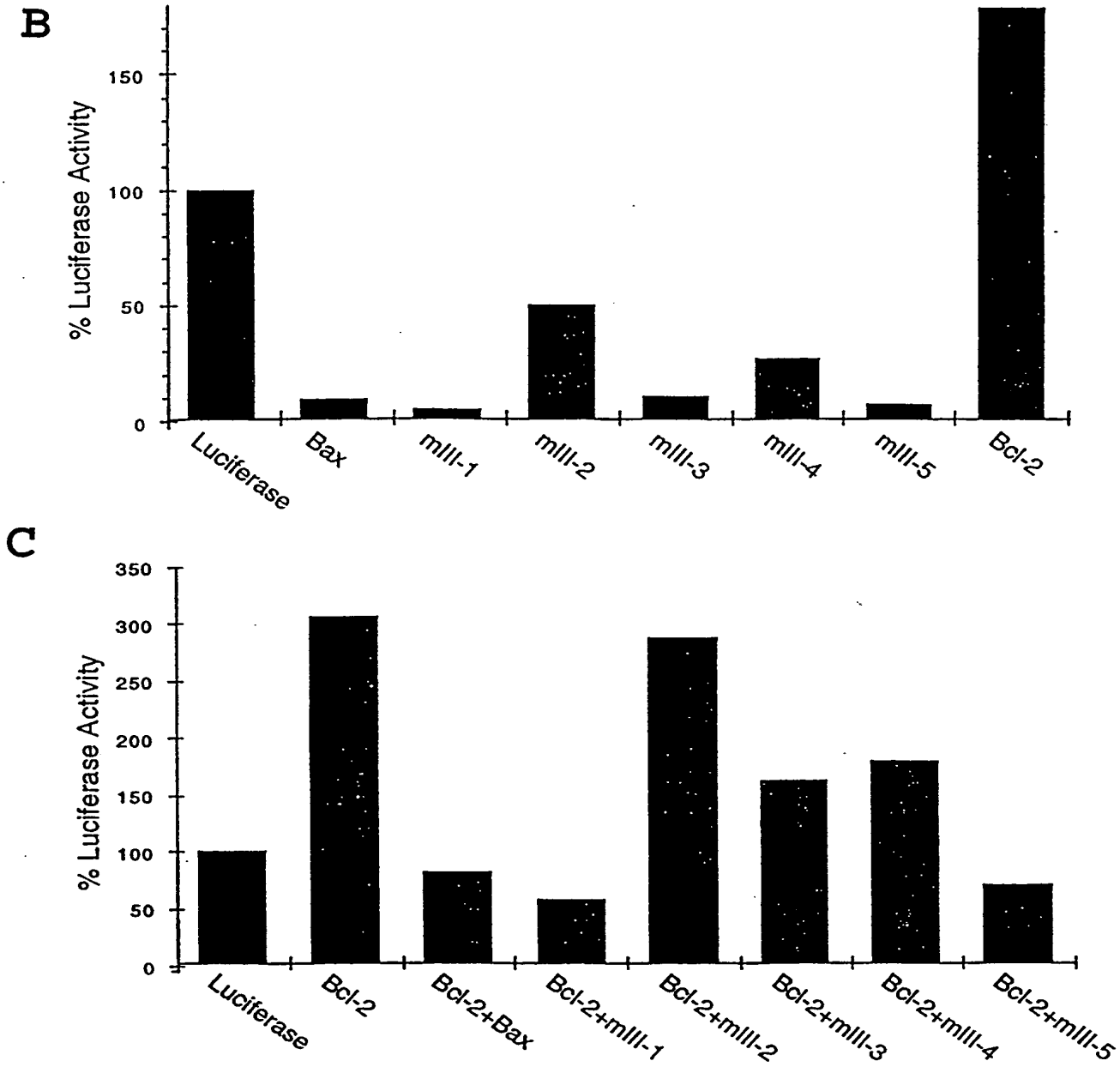


Figure 13

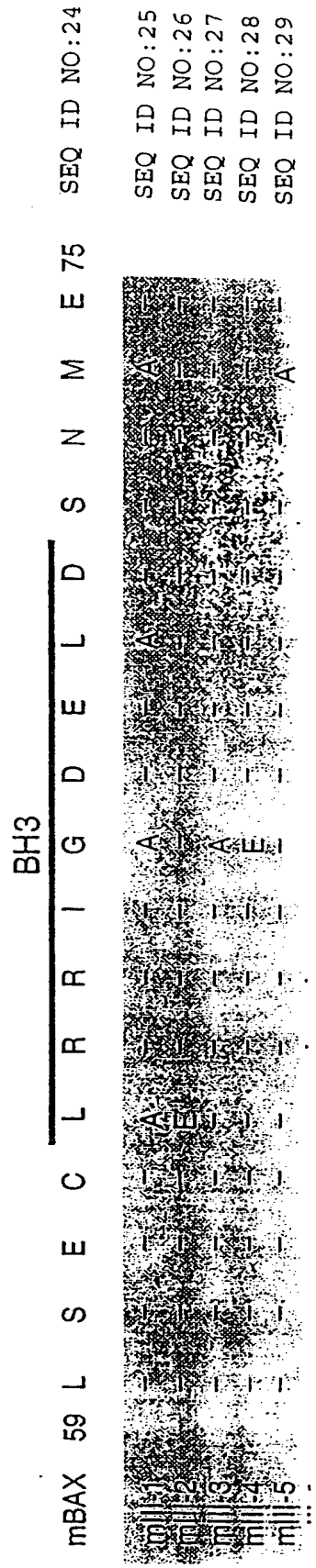

BH3
mBAX 59 L S E C L R R I G D E L D S N M E 75 SEQ ID NO:24
SEQ ID NO:25
SEQ ID NO:26
SEQ ID NO:27
SEQ ID NO:28
SEQ ID NO:29

Figure 13A

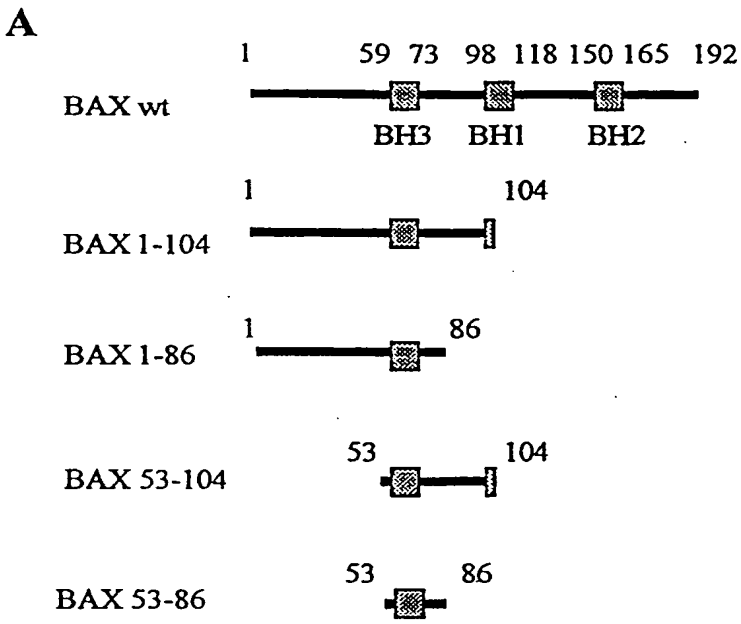

A
1
59 73 98 118 150 165 192
BAX wt
BH3 BH1 BH2
1
104
BAX 1-104
1
86
BAX 1-86
53
104
BAX 53-104
53
86
BAX 53-86
B
1
89 98
195
BID wt
BH3
1
73
BID 1-73
74
195
BID 74-195
74
128
BID 74-128
74
106
BID 74-106

Figure 14

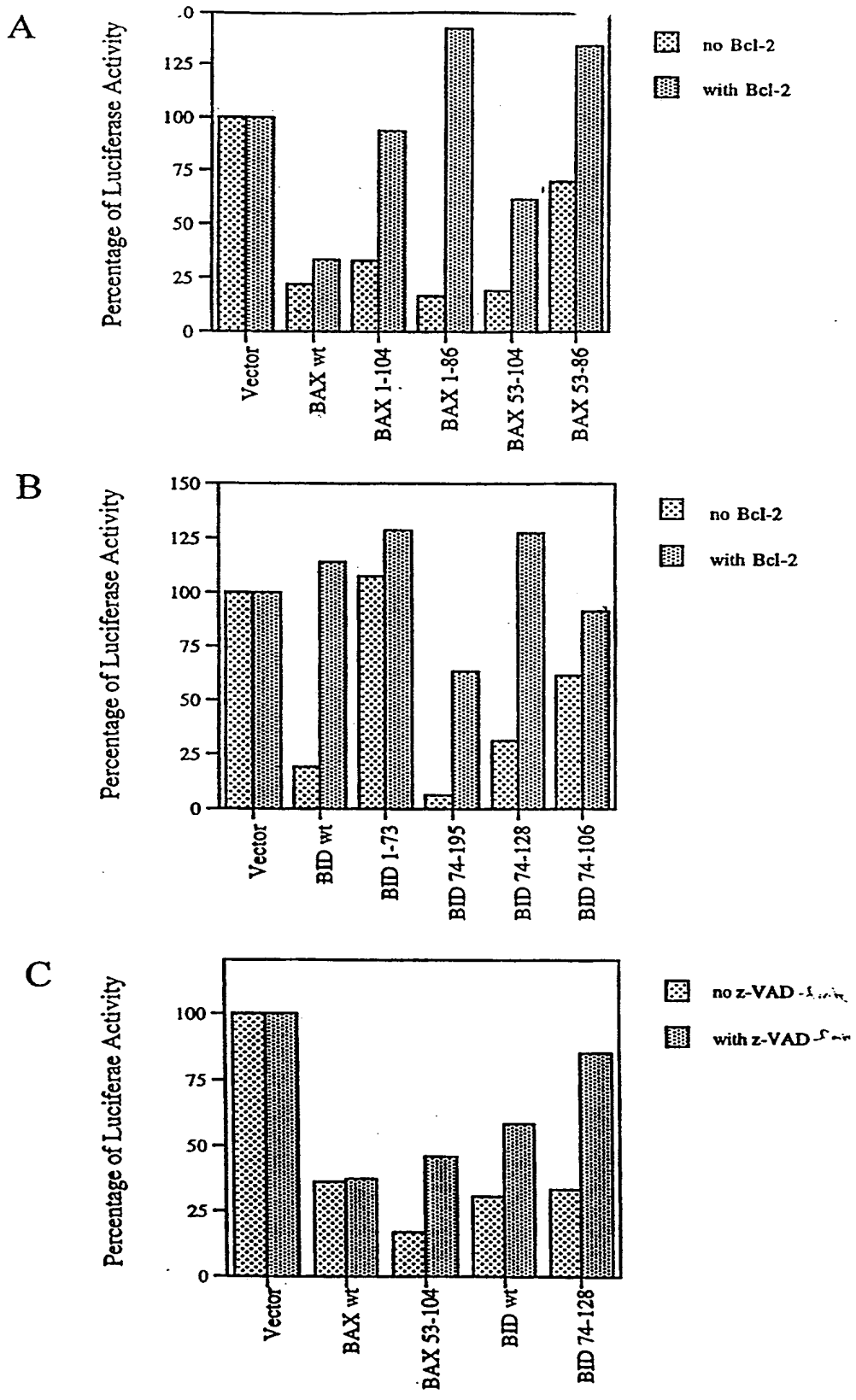

A
Percentage of Luciferase Activity
0
25
50
75
100
125
Vector
BAX wt
BAX 1-104
BAX 1-86
BAX 53-104
BAX 53-86
no Bcl-2
with Bcl-2
B
Percentage of Luciferase Activity
0
25
50
75
100
125
150
Vector
BID wt
BID 1-73
BID 74-195
BID 74-128
BID 74-106
no Bcl-2
with Bcl-2
C
Percentage of Luciferae Activity
0
25
50
75
100
Vector
BAX wt
BAX 53-104
BID wt
BID 74-128
no z-VAD
with z-VAD

Figure 15

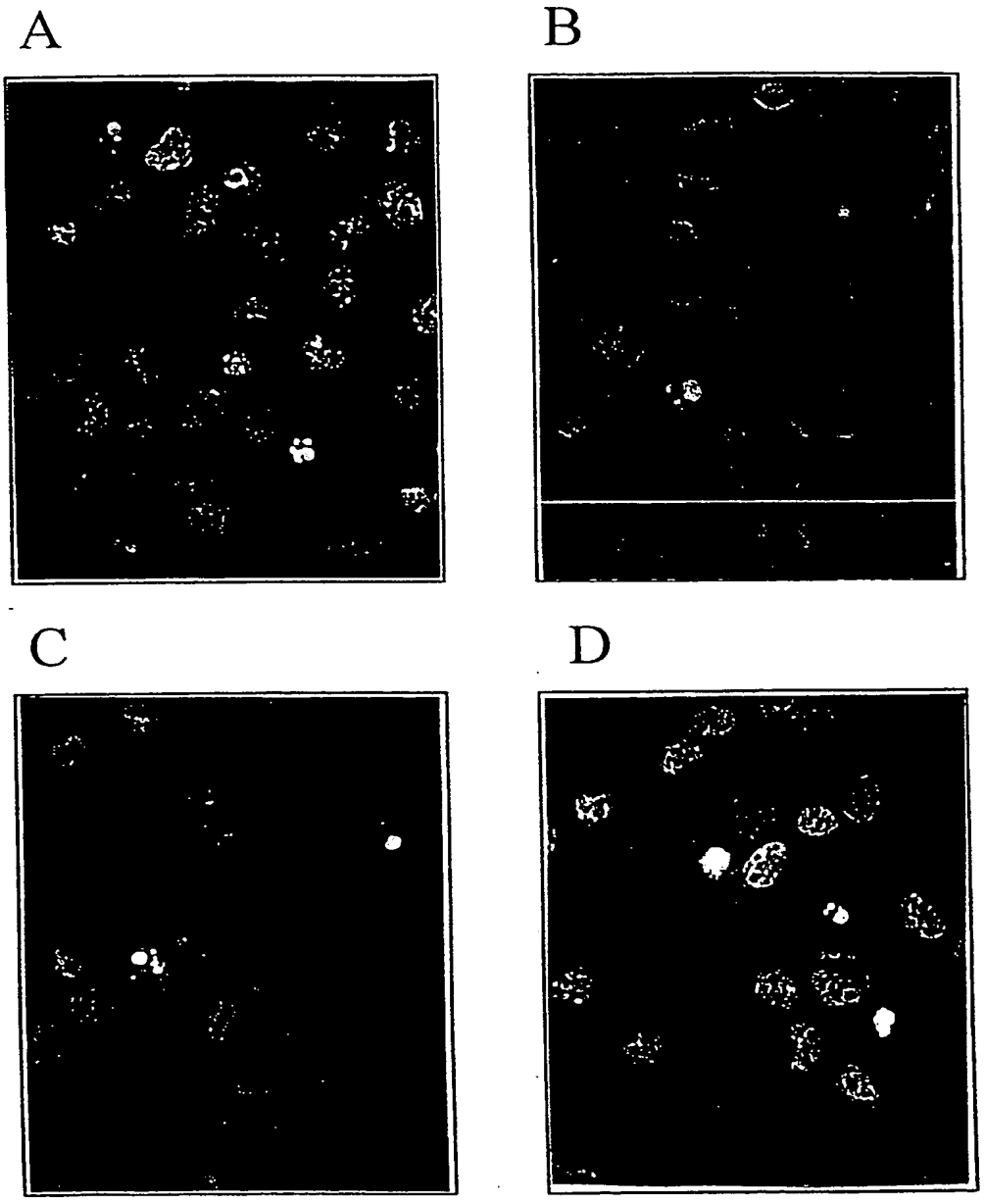
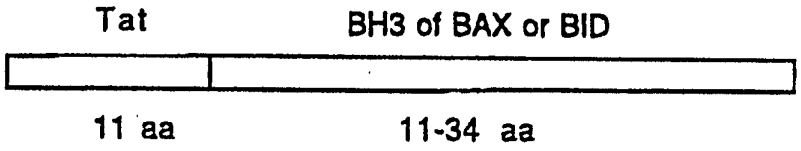


FIGURE 16



TAT PEPTIDE	YGRKKRRQRRR	SEQ ID NO:55
BAX (53-86):	DASTKKLSECLKRIGDELDSNMELQRMIAAVDTD	SEQ ID NO:30
BAX (53-76):	DASTKKLSECLKRIGDELDSNMEL	SEQ ID NO:31
BAX (63-76)M:	DASTKKLSECELDLKRIGDSNMEL	SEQ ID NO:32
BAX (57-71):	KKLSECLKRIGDELD	SEQ ID NO:33
BAX (57-71)M:	KKLSECELDLKRIGD	SEQ ID NO:34
BAX (61-71):	ECLKRIGDELD	SEQ ID NO:35
BID (75-106):	DSESQEEIIHNIARHLAQIGDEMDHNIQPTLV	SEQ ID NO:36
BID (81-100):	EIIHNIARHLAQIGDEMDHN	SEQ ID NO:37
BID (81-100)M:	EIIHNIARHQIGDEMDLAHN	SEQ ID NO:38
BID (84-98):	HNIARHLAQIGDEMD	SEQ ID NO:39

Figure 17A

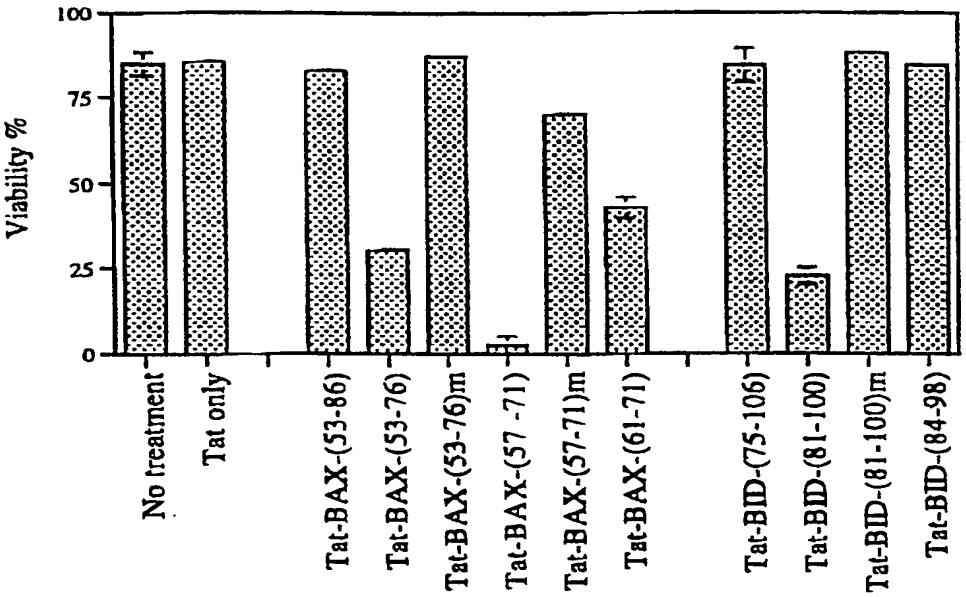
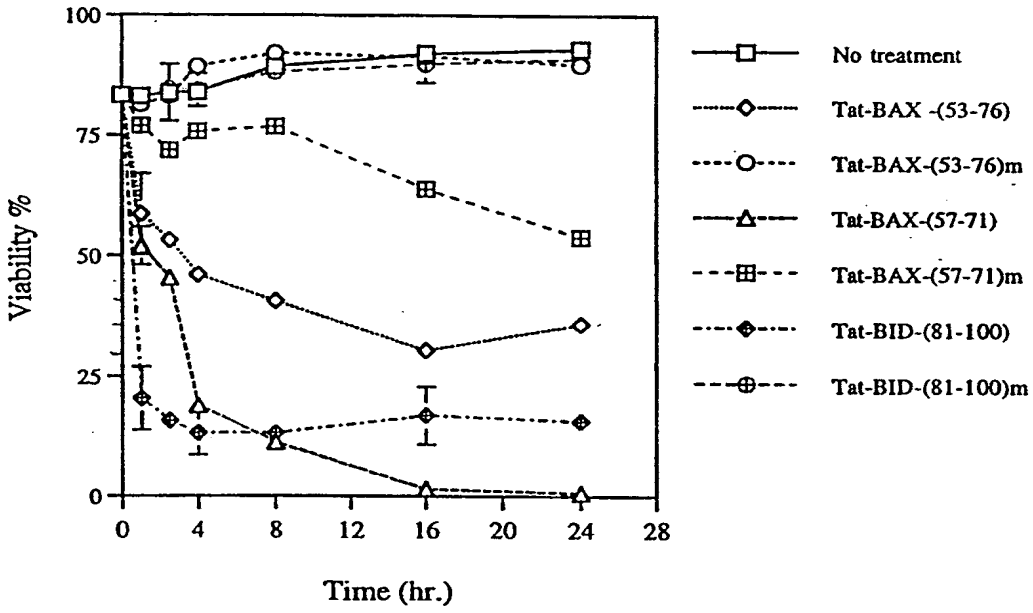

Viability %
100
75
50
25
0
No treatment
Tat only
Tat-BAX-(53-86)
Tat-BAX-(53-76)
Tat-BAX-(53-76)m
Tat-BAX-(57 -71)
Tat-BAX-(57-71)m
Tat-BAX-(61-71)
Tat-BID-(75-106)
Tat-BID-(81-100)
Tat-BID-(81-100)m
Tat-BID-(84-98)

Figure 17B

A


100
75
50
25
0
Viability %
0
4
8
12
16
20
24
28
Time (hr.)
No treatment
Tat-BAX -(53-76)
Tat-BAX-(53-76)m
Tat-BAX-(57-71)
Tat-BAX-(57-71)m
Tat-BID-(81-100)
Tat-BID-(81-100)m

B

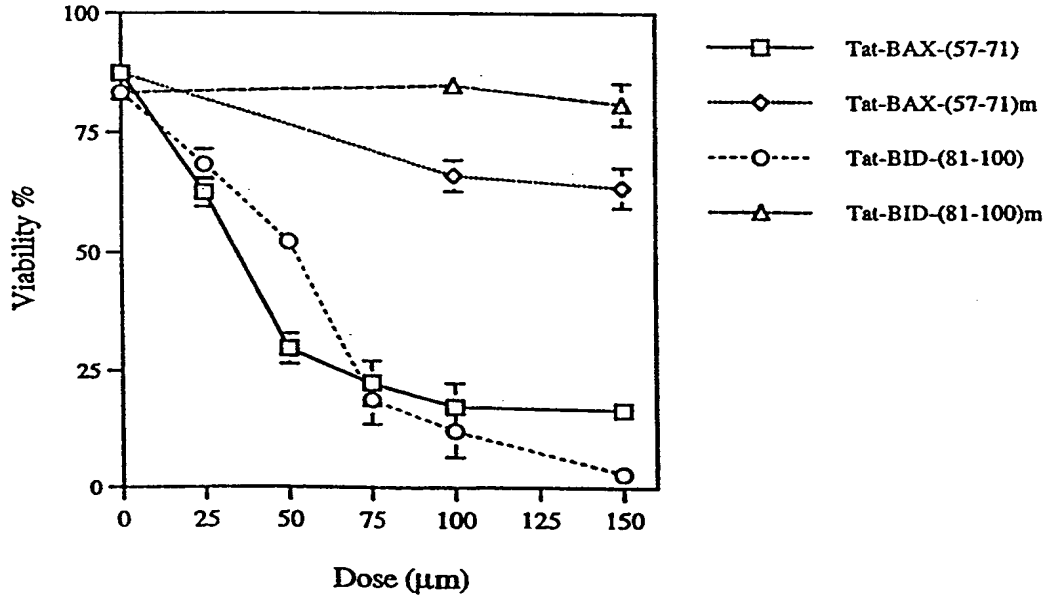

100
75
50
25
0
Viability %
0
25
50
75
100
125
150
Dose (µm)
Tat-BAX-(57-71)
Tat-BAX-(57-71)m
Tat-BID-(81-100)
Tat-BID-(81-100)m

Figure 18

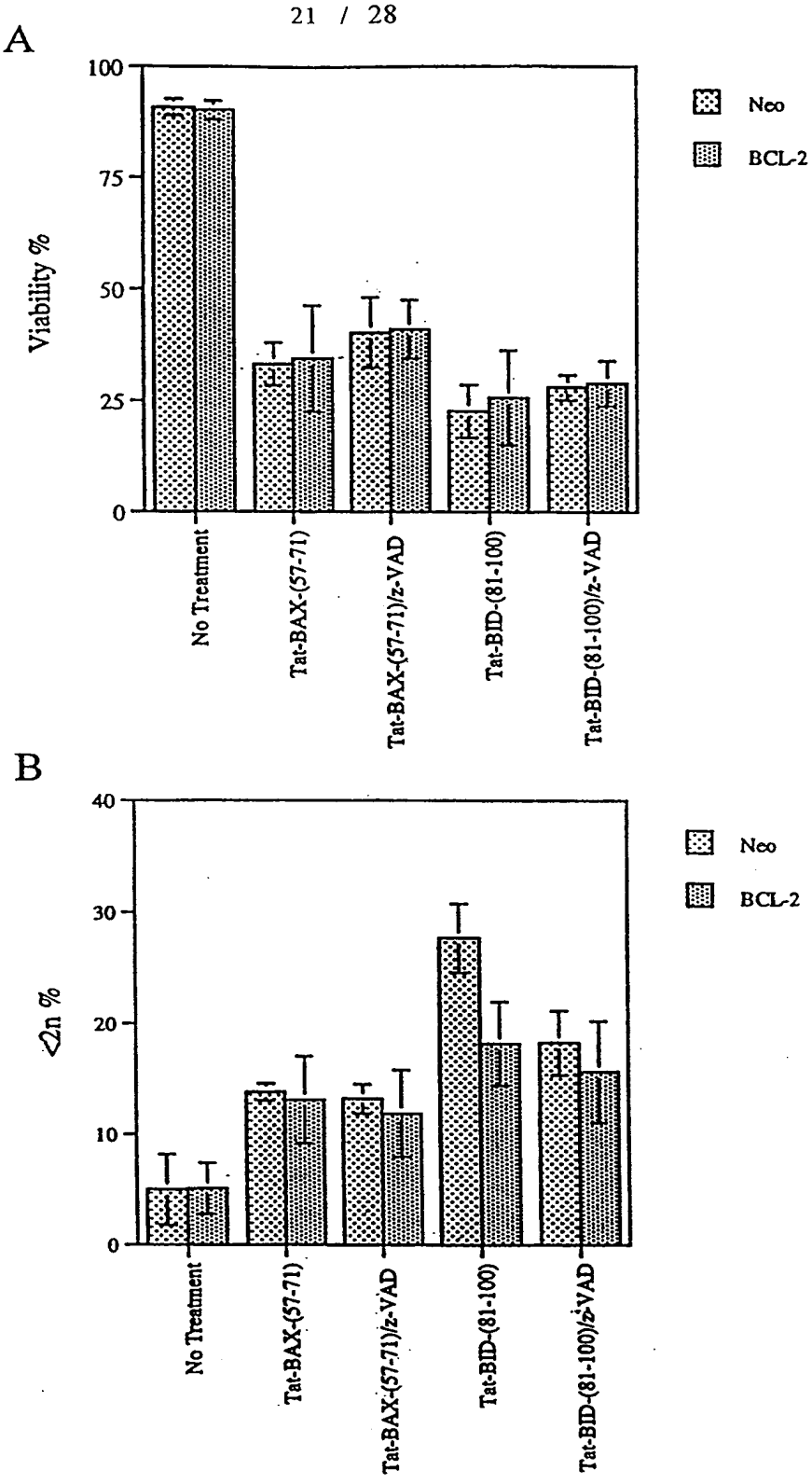

A
Viability %
100
75
50
25
0
Neo
BCL-2
No Treatment
Tat-BAX-(57-71)
Tat-BAX-(57-71)/z-VAD
Tat-BID-(81-100)
Tat-BID-(81-100)/z-VAD
B
<2n %
40
30
20
10
0
Neo
BCL-2
No Treatment
Tat-BAX-(57-71)
Tat-BAX-(57-71)/z-VAD
Tat-BID-(81-100)
Tat-BID-(81-100)/z-VAD

Figure 19

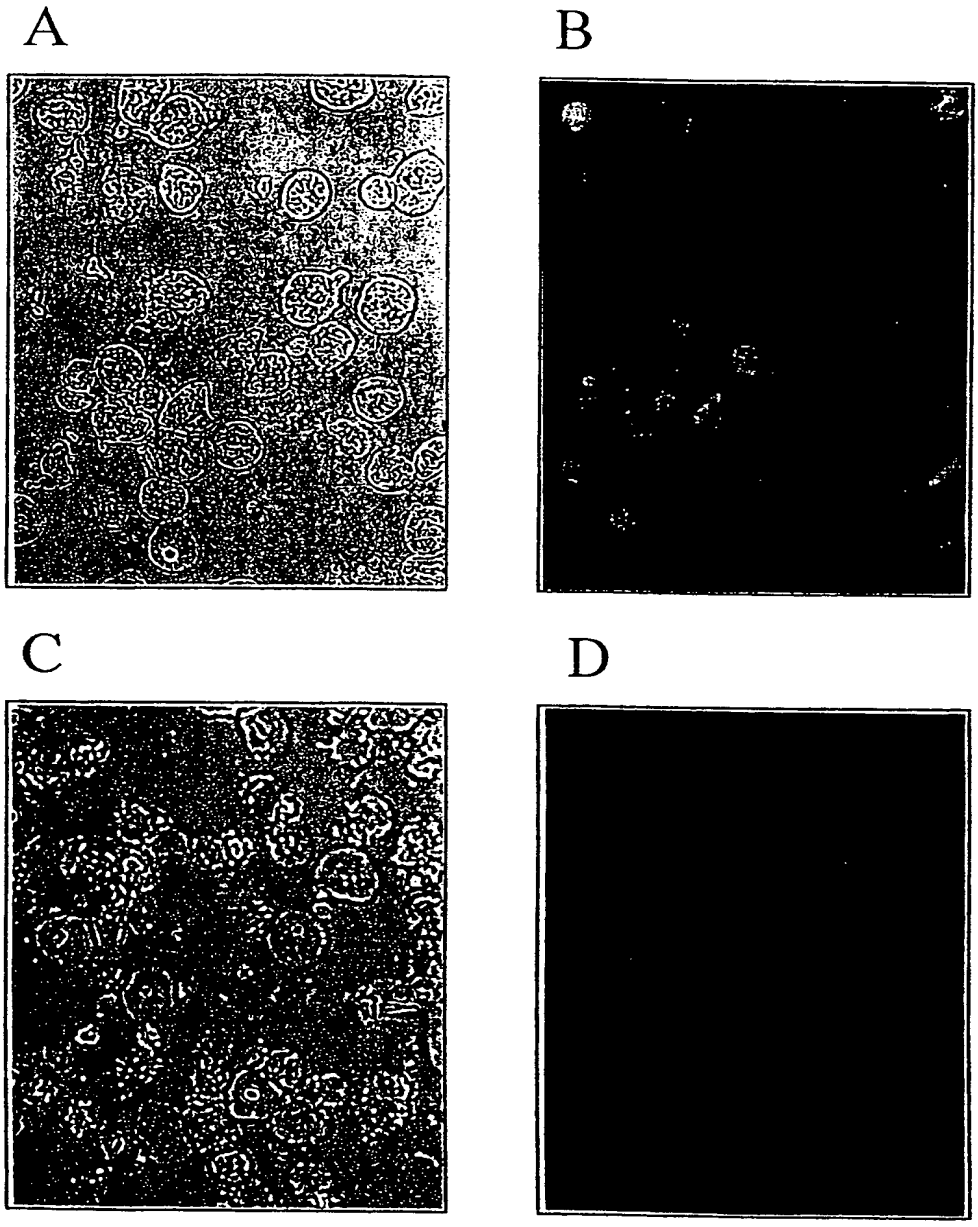


FIGURE 20

Murine BAD and Partial Human BAD sequences

```
mBAD    MGTPKQPSLAPAHALGLRKSDPGIRSLGSDAGGRRWRPAAQSMFQIPEFE   50

mBAD    PSEQEDASATDRGLGPSLTEDQPGPYLAPGLLGSNIHQQGRAATNSHHGG  100
                                                        |
                                                        G     1
hBAD

                                                  ________
mBAD    AGAMETRSRHSSYPAGTEEDEGMEEELSPFRGRSRSAPPNLWAAQRYGRE  150
        |||•| ||||||||||||||•|||| || ||||||||||||||||||||||
hBAD    AGAVEIRSRHSSYPAGTEDDEGMGEEPSPFRGRSRSAPPNLWAAQRYGRE   51

                                          ==========
mBAD    LRRMSDEFEGSFKGLPRPKSAGTATQMRQSAGWTRIIQSWWDRNLGKGGS  200
        ||||||||  ||
hBAD    LRRMSDEFVDSF                                         63
        ________
           BH3
                                                             204
mBAD    TPSQ
```

Figure 21A

B

Murine BAK sequence

MASGQGPGPPKVGCDESPSPSEQQVAQDTEEVFRSYVFYLHQQEQETQGRPPANPEMDNLPLEPNSIL
GQVGRQLALIGDDINRRYDTEFQNLLEQLQPTAGNAYELFTKIASSLFKSGISWGRVVALLGFGYRLA
LYVYQRGLTGFLGQVTCFLADIILHHYIARWIAQRGGWVAALNLRRDPILTVMVIFGVVLLGQFVVHR
FFRS

Human BAK sequence

MASGQGPGPPRQECGEPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEMVTLPLQPS
STMGQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGINWGRVVALLGFGY
RLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQRGGWVAALNLGNGPILNVLVVLGVVLLGQFV
VRRFFKS

C

Murine BAX sequence

MDGSGEQLGSGGPTSSEQIMKTGAFLLQGFIQDRAGRMAGETPELTLEQPPQDASTKKLSECLRRIGD
ELDSNMELQRMIADVDTDSPREVFFRVAADMFADGNFNWGRVVALFYFASKLVLKALCTKVPELIRTI
MGWTLDFLRERLLVWIQDQGGWEGLLSYFGTPTWQTVTIFVAGVLTASLTIWKKMG

Human BAX sequence

MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLSECLKRIGD
ELDSNMELQRMIAAVDTDSPREVFFRVAADMFSDGNFNWGRVVALFYFASKLVLKALCTKVPELIRTI
MGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGVLTASLTIWKKMG

Figure 21

```
huBid    - MDCEVNNGSSLRDECITNLLVFGFLQSCSDNSFRRELDALGHELPVLAPQ - 50
muBid    - MDSEVSNGSGLGAKHITDLLVFGFLQSSG--CTRQELEVLGRELPV-QAY - 47

huBid    - WEGY--DELQTDGNRSSHS-RLGRIEADSESQEDIIRNIARHLAQVGDSM - 97
muBid    - WEADLEDELQTDGSQASRSFNQGRIEPDSESQEEIIHNIARHLAQIGDEM - 97

huBid    - DRSIPPGLVNGLALQLRNTSRSEEDRNRDLATALEQLLQAYPRDMEKEKT -147
muBid    - DHNTQPTLVRQLAAQFMNGSLSEEDKRNCLAKALDEVKTAFPRDMENDKA -147

huBid    - MLVLALLLAKKVASHTPSLLRDVFHTTVNFINQNLRTYVRSLARNGMD   -195
muBid    - MLIMTMLLAKKVASHAPSLLRDVFHTTVNFINQNLFSYVRNLVRNEMD   -195
```

Figure 21D

Human BIK sequence

MSEVRPLSRDILMETLLYEQLLEPPTMEVLGMTDSEEDLDPMEDFDSLECMEGSDALALRLACIGDEMDVSLRAP
RLAQLSEVAMHSLGLAFIYDQTEDIRDVLRSFMDGFTTLKENIMRFWRSPNPGSWVSCEQVLLALLLLLALLLPL
LSGGLHLLLK

Figure 21E

Human BAD Partial Polynucleotide and Polypeptide Sequences

```
GGCGCTGGGGCTGTGGAGATCCGGAGTCGCCACAGCTCCTACCCCGCGGGGACGGAGGAC
60
 G  A  G  A  V  E  I  R  S  R  H  S  S  Y  P  A  G  T  E  D
20

GACGAAGGGATGGGGGAGGAGCCCAGCCCCTTTCGGGGCCGCTCGCGCTCGGCGCCCCCC
120
 D  E  G  M  G  E  E  P  S  P  F  R  G  R  S  R  S  A  P  P
40

AACCTCTGGGCAGCACAGCGCTATGGCCGCGAGCTCCGGAGGATGAGTGACGAGTTTGTG
180
 N  L  W  A  A  Q  R  Y  G  R  E  L  R  R  M  S  D  E  F  V
60

GACTCCTTT
189
 D  S  F
63
```

Figure 22A

Human BAK CDNA

```
   1 GAGGATCTAC AGGGGACAAG TAAAGGCTAC ATCCAGATGC CGGGAATGCA CTGACGCCCA
  61 TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC TGGGAGCAGC AGCCGCCAGC CCCTCGGACC
 121 TCCATCTCCA CCCTGCTGAG CCACCCGGGT TGGGCCAGGA TCCCGGCAGG CTGATCCCGT
 181 CCTCCACTGA GACCTGAAAA ATGGCTTCGG GGCAAGGCCC AGGTCCTCCC AGGCAGGAGT
 241 GCGGAGAGCC TGCCCTGCCC TCTGCTTCTG AGGAGCAGGT AGCCCAGGAC ACAGAGGAGG
 301 TTTTCCGCAG CTACGTTTTT TACCGCCATC AGCAGGAACA GGAGGCTGAA GGGGTGGCTG
 361 CCCCTGCCGA CCCAGAGATG GTCACCTTAC CTCTGCAACC TAGCAGCACC ATGGGGCAGG
 421 TGGGACGGCA GCTCGCCATC ATCGGGGACG ACATCAACCG ACGCTATGAC TCAGAGTTCC
 481 AGACCATGTT GCAGCACCTG CAGCCCACGG CAGAGAATGC CTATGAGTAC TTCACCAAGA
 541 TTGCCACCAG CCTGTTTGAG AGTGGCATCA ATTGGGGCCG TGTGGTGGCT CTTCTGGGCT
 601 TCGGCTACCG TCTGGCCCTA CACGTCTACC AGCATGGCCT GACTGGCTTC CTAGGCCAGG
 661 TGACCCGCTT CGTGGTCGAC TTCATGCTGC ATCACTGCAT TGCCCGGTGG ATTGCACAGA
 721 GGGGTGGCTG GGTGGCAGCC CTGAACTTGG GCAATGGTCC CATCCTGAAC GTGCTGGTGG
 781 TTCTGGGTGT GGTTCTGTTG GGCCAGTTTG TGGTACGAAG ATTCTTCAAA TCATGACTCC
 841 CAAGGGTGCC CTTTGGGTCC CGGTTCAGAC CCCTGCCTGG ACTTAAGCGA AGTCTTTGCC
 901 TTCTCTGTTC CCTTGCAGGG TCCCCCCTCA AGAGTACAGA AGCTTTAGCA AGTGTGCACT
 961 CCAGCTTCGG AGGCCCTGCG TGGGGGCCAG TCAGGCTGCA GAGGCACCTC AACATTGCAT
1021 GGTGCTAGTG CCCTCTCTCT GGGCCCAGGG CTGTGGCCGT CTCCTCCCTC AGCTCTCTGG
1081 GACCTCCTTA GCCCTGTCTG CTAGGCGCTG GGGAGACTGA TAACTTGGGG AGGCAAGAGA
1141 CTGGGAGCCA CTTCTCCCCA GAAAGTGTTT AACGGTTTTA GCTTTTTATA ATACCCTTGT
1201 GAGAGCCCAT TCCCACCATT CTACCTGAGG CCAGGACGTC TGGGGTGTGG GGATTGGTGG
1261 GTCTATGTTC CCCAGGATTC AGCTATTCTG GAAGATCAGC ACCCTAAGAG ATGGGACTAG
1321 GACCTGAGCC TGGTCCTGGC CGTCCCTAAG CATGTGTCCC AGGAGCAGGA CCTACTAGGA
1381 GAGGGGGGCC AAGGTCCTGC TCAACTCTAC CCCTGCTCCC ATTCCTCCCT CCGGCCATAC
1441 TGCCTTTGCA GTTGGACTCT CAGGGATTCT GGGCTTGGGG TGTGGGGTGG GGTGGAGTCG
1501 CAGACCAGAG CTGTCTGAAC TCACGTGTCA GAAGCCTCCA AGCCTGCCTC CCAAGGTCCT
1561 CTCAGTTCTC TCCCTTCCTC TCTCCTTATA GACACTTGCT CCCAACCCAT TCACTACAGG
1621 TGAAGGCTCT CACCCATCCC TGGGGGCCTT GGGTGAGTGG CCTGCTAAGG CTCCTCCTTG
1681 CCCAGACTAC AGGGCTTAGG ACTTGGTTTG TTATATCAGG GAAAAGGAGT AGGGAGTTCA
1741 TCTGGAGGGT TCTAAGTGGG AGAAGGACTA TCAACACCAC TAGGAATCCC AGAGGTGGAT
1801 CCTCCCTCAT GGCTCTGGCA CAGTGTAATC CAGGGGTGTA GATGGGGGAA CTGTGAATAC
1861 TTGAACTCTG TTCCCCCACC CTCCATGCTC CTCACCTGTC TAGGTCTCCT CAGGGTGGGG
1921 GGTGACAGTG CCTTCTCTAT TGGCACAGCC TAGGGTCTTG GGGGTCAGGG GGGAGAAGTT
1981 CTTGATTCAG CCAAATGCAG GGAGGGGAGG CAGATGGAGC CCATAGGCCA CCCCCTATCC
2041 TCTGAGTGTT TGGAAATAAA CTGTGCAATC CCCTCAAAAA AAAAACGGAG ATCC
```

Figure 22B

C Human BAX sequence

```
   1 ATGGACGGGT CCGGGGAGCA GCCCAGAGGC GGGGGGCCCA CCAGCTCTGA GCAGATCATG
  61 AAGACAGGGG CCCTTTTGCT TCAGGGTTTC ATCCAGGATC GAGCAGGGCG AATGGGGGGG
 121 GAGGCACCCG AGCTGGCCCT GGACCCGGTG CCTCAGGATG CGTCCACCAA GAAGCTGAGC
 181 GAGTGTCTCA AGCGCATCGG GGACGAACTG GACAGTAACA TGGAGCTGCA GAGGATGATT
 241 GCCGCCGTGG ACACAGACTC CCCCCGAGAG GTCTTTTTCC GAGTGGCAGC TGACATGTTT
 301 TCTGACGGCA ACTTCAACTG GGGCCGGGTT GTCGCCCTTT TCTACTTTGC CAGCAAACTG
 361 GTGCTCAAGG CCCTGTGCAC CAAGGTGCCG GAACTGATCA GAACCATCAT GGGCTGGACA
 421 TTGGACTTCC TCCGGGAGCG GCTGTTGGGC TGGATCCAAG ACCAGGGTGG TTGGGACGGC
 481 CTCCTCTCCT ACTTTGGGAC GCCCACGTGG CAGACCGTGA CCATCTTTGT GGCGGGAGTG
 541 CTCACCGCCT CGCTCACCAT CTGGAAGAAG ATGGGCTGA
```

D Human BID Sequence

```
   1 ATGGACTGTG AGGTCAACAA CGGTTCCAGC CTCAGGGATG AGTGCATCAC
AAACCTACTG
  61 GTGTTTGGCT TCCTCCAAAG CTGTTCTGAC AACAGCTTCC GCAGAGAGCT
GGACGCACTG
 121 GGCCACGAGC TGCCAGTGCT GGCTCCCCAG TGGGAGGGCT ACGATGAGCT
GCAGACTGAT
 181 GGCAACCGCA GCAGCCACTC CCGCTTGGGA AGAATAGAGG CAGATTCTGA
AAGTCAAGAA
 241 GACATCATCC GGAATATTGC CAGGCACCTC GCCCAGGTCG GGGACAGCAT
GGACCGTAGC
 301 ATCCCTCCGG GCCTGGTGAA CGGCCTGGCC CTGCAGCTCA GGAACACCAG
CCGGTCGGAG
 361 GAGGACCGGA ACAGGGACCT GGCCACTGCC CTGGAGCAGC TGCTGCAGGC
CTACCCTAGA
 421 GACATGGAGA AGGAGAAGAC CATGCTGGTG CTGGCCCTGC TGCTGGCCAA
GAAGGTGGCC
 481 AGTCACACGC CGTCCTTGGC TCCGTGATGT CTTTCACACA ACAGTAATTT
TATTAACCAG
 541 AACCTACGCA CCTACGTGAG GAGCTTAGCC AGAAATGGGA TGGACTGA
```

E Human BIK Sequence

```
   1 CAGCATCGCC GCCGCCAGAG GAGAAATGTC TGAAGTAAGA CCCCTCTCCA GAGACATCTT
  61 GATGGAGACC CTCCTGTATG AGCAGCTCCT GGAACCCCCG ACCATGGAGG TTCTTGGCAT
 121 GACTGACTCT GAAGAGGACC TGGACCCTAT GGAGGACTTC GATTCTTTGG AATGCATGGA
 181 GGGCAGTGAC GCATTGGCCC TGCGGCTGGC CTGCATCGGG GACGAGATGG ACGTGAGCCT
 241 CAGGGCCCCG CGCCTGGCCC AGCTCTCCGA GGTGGCCATG CACAGCCTGG GTCTGGCTTT
 301 CATCTACGAC CAGACTGAGG ACATCAGGGA TGTTCTTAGA AGTTTCATGG ACGGTTTCAC
 361 CACACTTAAG GAGAACATAA TGAGGTTCTG GAGATCCCCG AACCCCGGGT CCTGGGTGTC
 421 CTGCGAACAG GTGCTGCTGG CGCTGCTGCT GCTGCTGGCG CTGCTGCTGC CGCTGCTCAG
 481 CGGGGGCCTG CACCTGCTGC TCAAGTGAGC CCCCGGCGGC TCAGGCGTGG CTGGCCCCAC
 541 CCCCATGACC ACTGCCCTGA GGTGGCGGCC TGCTGCTGTT ATCTTTTTAA CTGTTTTCTC
 601 ATGATGCCTT TTATATTAAC CCCGTGATAG TGCTGGAACA CTGCTGAGGT TTTATACTCA
 661 GGTTTTTTGT TTTTTTTTTA TTCCAGTTTT CGTTTTTTCT AAAAGATGAA TTCCTATGGC
 721 TCTGCAATTG TCACCGGTTA ACTGTGGCCT GTGCCCAGGA AGAGCCATTC ACTCCTGCCC
 781 CTGCCCCACAC GGCAGGTAGC AGGGGGAGTG CTGGTCACAC CCCTGTGTGA TATGTGATGC
 841 CCTCGGCAAA GAATCTACTG GAATAGATTC CGAGGAGCAG GAGTGCTCAA TAAAATGTTG
 901 GTTTCCAGCA AAAAAAAAAA AAA
```

Figure 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/2; 530/300; 536/23.1, 23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/300; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DNA and amino acid databases
BH3 domain, SEQ ID NO: 1, 3, 5, 7, 9, 31, 33, 35, 37, 40, 55, Tat peptide, BCL-2 family, apoptosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,656,725 A (CHITTENDEN et al) 12 August 1997, see entire document.	1-4, 6, 8-11, 13-17, 19-21 --- 5, 7, 12, 18
X	BOYD et al. Bik, A Novel Death-Inducing Protein Shares a Distinct Sequence Motif with Bcl-2 Family Proteins and Interacts with Viral and Cellular Survival-Promoting Proteins. Oncogene. 1995, Vol. 11, pages 1921-1928, see entire document.	21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
17 DECEMBER 1998	26 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY A. JOHNSON Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHITTENDEN et al. A Conserved Domain in Bak, Distinct from BH1 and BH2, Mediates Cell Death and Protein Binding Functions. EMBO. 1995, Vol. 14, pages 5589-5596, see entire document.	21
X --- Y	WANG et al. BID: A Novel BH3 Domain-Only Death Agonist. Genes and Development. 1996, Vol. 10, pages 2859-2869, see entire document.	21 ----- 5, 12, 18
Y	US 5,652,122 A (FRANKEL et al) 29 July 1997, see abstract and SEQ ID NO:1.	7

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C07K 7/00, 14/00; C07H 21/04, 21/02; C12N 15/11; A61K 38/04, 38/16

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CELL DEATH AGONISTS

(57) Abstract

Small polypeptides and peptides of 5 to 50 amino acids having cell death agonist activity are provided. The polypeptides are at least 9 amino acids in length and contain the BH3 domain of a pro–apoptotic BCL–2 family member. The peptides contain 5 to 8 amino acids from the BH3 domain. Methods of promoting apoptosis with these cell death agonist polypeptides and peptides and their encoding polynucleotides are also provided.

*(Referred to in PCT Gazette No. 25/1999, Section II)

BNSDOCID: <WO_____9916787A1_IA>

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SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
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TG	Togo
TJ	Tajikistan
TM	Turkmenistan
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UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

CELL DEATH AGONISTS

Cross-Reference to Related Applications

This application claims the benefit of, and incorporates herein by reference, the U.S. Provisional Application entitled "BH3 Domain of Bad is Required for Heterodimerization with BCL-X_L and Pro-Apoptotic Activity", which was filed September 26, 1997 as Attorney Docket No. 6029-1985.

Reference to Government Grant

This invention was made with government support under Grant Number R01 #50239. The government has certain rights in this invention.

Background of the Invention

(1) Field of the Invention

This invention relates generally to the regulation of apoptosis and to compounds which regulate apoptosis, and more particularly, to a novel cell death agonist.

(2) Description of the Related Art

Programmed cell death, referred to as apoptosis, plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms (Raff, *Nature 356*:397-400, 1992). Genetic and molecular analysis from nematodes to humans has indicated that the apoptotic pathway of cellular suicide is highly conserved (Hengartner and Horvitz, *Cell 76*:1107-1114, 1994) In addition to being essential for normal development and maintenance, apoptosis is important in the defense against viral infection and in preventing the emergence of cancer.

The BCL-2 family of proteins constitutes an intracellular checkpoint of apoptosis. The founding member of this family is the apoptosis-inhibiting protein encoded by the *bcl-2* protooncogene which was initially isolated from a follicular lymphoma (Bakhshi et al., *Cell 41*:889-906, 1985; Tsujimoto et al, *Science 229*:1390-1393, 1985; Cleary and Sklar, *Proc Natl Acad Sci USA 82*:7439-7443, 1985). The BCL-2 protein is a 25 kD, integral membrane protein localized to intracellular membranes including mitochondria. This factor extends survival in many different cell types by inhibiting apoptosis elicited by a variety of death-inducing stimuli (Korsmeyer, *Blood 80*:879-886, 1992).

The family of BCL-2-related proteins is comprised of both anti-apoptotic and pro-apoptotic members that function in a distal apoptotic pathway common to all multi-cellular organisms. It has been suggested that the ratio of anti-apoptotic (BCL-2, BCL-X_L, MCL-1 and A1) to pro-apoptotic (BAX, BAK, BCL-X_S, BAD, BIK and BID) molecules dictates whether a cell will respond to a proximal apoptotic stimulus. (Oltvai et al., *Cell 74*:609-619, 1993; Farrow, et al., *Curr. Opin. Gen. Dev. 6*: 45-49, 1996). Because members of this family can form both homodimers and heterodimers, the latter often between anti- and pro-apoptotic polypeptides, the balance

of these homodimers and heterodimers could play a role in regulating apoptosis (Oltvai and Korsmeyer, *Cell* 79:189-192, 1994).

Members of the BCL-2 family have been defined by sequence homology that is largely based upon conserved motifs termed BCL-Homology domains. (Yin et al, *Nature* 369:321-323, 1994). BCL-Homology domains 1 and 2 (BH1 and BH2) have been shown to be important in dimerization and in modulating apoptosis (Yin et al., *supra*). A third homology region, BH3, has been found in some family members and shown to be important in dimerization as well as promoting apoptosis (Boyd et al., *Oncogene 11*:1921-1928; Chittenden et al., *Embo J 14*:5589-5596, 1995). BH4, the most recently identified homology domain, is present near the amino terminal end of some pro-apoptotic family members (Farrow et al., *supra*).

The BH3 domain may play a role in the promotion of death by full-length pro-apoptotic family members, although BAD was not heretofore known to contain a BH3 domain. For example, the pro-apoptotic family member BCL-X_S, which is translated from an alternatively spliced version of the mRNA encoding BCL-X_L, contains BH3 and BH4 domains, but lacks BH1 and BH2 domains. BCL-X_S inhibits the ability of BCL-2 to enhance the survival of growth-factor deprived cells (Boise et al. *Cell* 74:597-608, 1993). BIK and BID are other death promoting BCL-2 family members having a BH3 but not BH1 or BH2 domains and which also lack a BH4 domain (Boyd et al., *Oncogene 11*:1921-1928, 1995; Wang et al., *Nature* 379:554-556, 1996).

Deletion analysis has indicated that the BH3 domain of the pro-apoptotic family members BAK, BAX, and BIK is required for them to heterodimerize with BCL-X_L or BCL-2 and also to promote cell death (Chittenden et al., *Embo J 14*:5589-5596, 1995; Zha et al., *supra*). For example, a significant loss of viability was observed in

cells transiently transfected with a plasmid expressing a 51 amino acid BAK polypeptide which contained BH3 but lacked BH1 and BH2 (Chittenden et al., *supra*). However, a BH3-containing 46 amino acid fragment of BAK, which bound to BCL-X_L both *in vitro* and in transfected cells, was reported to exhibit no cell killing activity unless the BAK hydrophobic tail element was attached (Chittenden et al., *supra*).

Other mutagenesis studies revealed that pro-apoptotic BID also interacts with BCL-2, BCL-X_L, and BAX through its BH3 domain and indicated that the corresponding binding site on these partner proteins is the BH1 domain, and perhaps also the BH2 domain (Wang et al., *supra*.) These data in combination with the predicted three-dimensional structures of BCL-2 and BAX, which are similar to the solved structure of BCL-X_L (Muchmore et al., *Nature 381*:335-341, 1996), were suggested to support a hypothesis that a BH3-BH1 mediated interaction between BID and a partner protein would occur by binding of the amphipathic α-helix of BID's BH3 domain to the exposed hydrophobic cleft contributed by the BH1 domain of the partner protein (Wang et al., *supra*).

A recent article described the three-dimensional structure of a complex between full-length BCL-X_L and a 16 amino acid Bak peptide (BAK 72-87) containing the BH3 domain (Sattler et al., *Science 175*:983-986, 1997). The BAK peptide, which is a random coil in solution, forms an α helix upon binding in a hydrophobic cleft formed by the BH1, BH2, and BH3 regions of BCL-X_L, with certain hydrophobic side chains of the BAK peptide (Val^{74}, Leu^{78}, and Ile^{81}) pointing into the cleft and certain charged side chains of the peptide (Arg^{76}, Asp^{83}, and Asp^{84}) being close to oppositely charged residues of BCL-X_L. Smaller BAK peptides from this region, including an 11mer peptide corresponding to BAK residues 77 to 87, reportedly did not bind to BCL-X_L.

However, BH3-BH1 binding may not be involved in all interactions between BCL-2 related proteins. For example, pro-apoptotic BIK and BCL-X_S, both of which lack the BH1 and BH2 domains, have been shown to interact (Boyd et al., *supra*). In addition, it has been demonstrated that BAX does not require BH1 or BH2 to homodimerize (Zha et al., *supra*).

Some disease conditions are believed to be related to the development of a defective down-regulation of apoptosis in the affected cells. For example, neoplasias may result, at least in part, from an apoptosis-resistant state in which cell proliferation signals inappropriately exceed cell death signals. Furthermore, some DNA viruses such as Epstein-Barr virus, African swine fever virus and adenovirus, parasitize the host cellular machinery to drive their own replication and at the same time modulate apoptosis to repress cell death and allow the target cell to reproduce the virus. Moreover, certain disease conditions such as lymphoproliferative conditions, cancer including drug resistant cancer, arthritis, inflammation, autoimmune diseases and the like may result from a down regulation of cell death regulation. In such disease conditions it would be desirable to promote apoptotic mechanisms.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

Summary of the Invention

In accordance with the present invention, it has been discovered that relatively short polypeptides including a BH3 domain derived from a pro-apoptotic

member of the BCL-2 family can promote apoptosis. Such polypeptides are shorter than the full length of the family member from which it is derived. The term "pro-apoptotic BCL-2 family member" refers to any polypeptide having a BH3 domain as defined herein and having the ability to promote cell death in one or more of the assays described herein. Pro-apoptotic family members include BAD, BAK, BAX, BID, and BIK.

The present invention is based on the discovery reported herein (1) that BAD (Bcl-2 Associated cell Death promoter) has a BH3 domain which is essential for apoptotic function and (2) that the BH3 domain of any pro-apoptotic member of the BCL-2 family is sufficient to promote apoptosis. In particular, the inventor has discovered that small polypeptides of 50 or fewer amino acids comprising the 9 amino acid BH3 domain have significant death agonist activity when administered to cells. This discovery was unexpected because it was not previously known that all BCL-2 pro-apoptotic family members contain a BH3 domain, nor was it known that a polypeptide containing the BH3 domain of any pro-apoptotic member is sufficient to promote apoptosis.

Accordingly, one aspect of the present invention provides a polypeptide containing a bcl-homology domain 3 (BH3 polypeptide) of from about 9 to about 50 contiguous amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The BH3 domain comprises a nine amino acid sequence as set forth in SEQ ID NO:40 (Leu-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Asp-Xaa_5-Xaa_6-Xaa_7, wherein Xaa_1 is Arg or Ala, Xaa_2 is Arg, Ile, Leu, Lys, Gln or Cys, Xaa_3 is Met, Ile or Val, Xaa_4 is Ser or Gly, Xaa_5 is Glu, Asp or Ser, Xaa_6 is Phe, Ile, Leu or Met, and Xaa_7 is Val, Glu, Asn or Asp), or a conservatively substituted variant thereof, and which is identified more particularly by homology to the sequences shown in FIG. 1 (SEQ ID NO:1-9). In preferred

embodiments, the BH3 domain is identical to or is a conservatively substituted variant of a BH3 domain from a human or murine BAD, BAK, BAX, BID, or BIK polypeptide. In one embodiment, the BH3 polypeptide is operably linked to a cell penetrating agent.

Another aspect of the invention provides a BH3 domain peptide having death agonist activity which comprises between about five to eight contiguous amino acids from the BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof.

Yet another aspect of the invention provides polynucleotides encoding a BH3 polypeptide of no more than 50 amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The invention also provides polynucleotides encoding BH3 domain peptides of about five to eight contiguous amino acids from SEQ ID NO:40, or a conservatively substituted variant thereof. These polynucleotide may be used to transfect a target cell for expression of the BH3 polypeptide to promote death of the target cell.

In other embodiments, the present invention provides a method for promoting apoptosis in a target cell comprising administering to the cell a death-promoting amount of a BH3 polypeptide or a BH3 domain peptide. The BH3 polypeptide comprises no more than 50 contiguous amino acids having cell death agonist activity and comprising a BH3 domain of a pro-apoptotic BCL-2 family member, while the BH3 domain peptide has cell death agonist activity and comprises five to eight contiguous amino acids of the BH3 domain. In one embodiment, the BH3 polypeptide or BH3 domain peptide is operably linked to a cell-penetrating agent which improves entry of the BH3 polypeptide into the cell. Alternatively, the BH3 polypeptide or BH3 domain peptide can be administered to the target cell by transfecting

the cell with an expression vector which comprises a polynucleotide encoding the BH3 polypeptide or BH3 domain peptide.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of new BH3 polypeptides which are relatively short in length and which possess cell death agonist activity; the provision of peptides from the BH3 domain, the provision of polynucleotides encoding these polypeptides and peptides; the provision of BH3 polypeptide compositions and peptide compositions having cell death agonist activity and which can be readily delivered intracellularly to produce a death agonist activity; and the provision of a method for promoting death of a target cell with these compositions.

Brief Description of the Drawings

Figure 1 illustrates the amino acid sequences of the BH3 domains from human (h) and murine (m) BAD, BAK, BAX, BIK, and BID (SEQ ID NO:1-9);

Figure 2 illustrates the structures of BCL-2 family members showing the locations of the homology domains relative to the N-terminus as BH4, BH3, BH1, and BH2, with TM representing the hydrophobic transmembrane C-terminal tail present in most members;

Figure 3 illustrates that BAD has a BH1/BH3 region that is required for cell death and heterodimerization with BCL-2 showing (A) a map of a nested set of BAD deletion mutants indicating retained amino acids and the position of the BH1/BH3 and BH2 domains and (B) the binding of P^{32}-labeled GST-BCL-2 to these BAD deletion mutants transferred to nitrocellulose (upper panel) from a SDS-PAGE gel (lower panel);

Figure 4 illustrates aligned partial sequences of human and murine BAD, BAK, BAX, BID, and BIK (SEQ ID

NO:10-18) showing the sequence homology within BH3 domains (underlined) with identical amino acids boxed;

Figure 5 illustrates the predicted three-dimensional amphipathic α-helix structure of the BAD BH3 domain showing views of the hydrophobic surface (left) and polar surface (right) with the locations of the hydrophobic and polar amino acids forming each surface identified;

Figure 6 illustrates that the BAD BH1/BH3 domain is essential for pro-apoptotic function showing (A) the structure of BAD deletion mutants indicating retained amino acids and positions of the BH1/BH3 and BH2 domains, (B) the apoptosis-promoting activity of these BAD deletion mutants as measured by transient co-transfection with a luciferase reporter vector into BAD-deficient murine embryonic fibroblasts, and (C) the BCL-2 or BCL-X_L binding ability of these BAD deletion mutants in an *in vitro* binding assay;

Figure 7 illustrates the effect of BAD BH3 mutations on heterodimerization of BAD with BCL-2 or BCL-X_L showing (A) ^{35}S-labeled wild-type (WT) and mutant BAD proteins substituted with alanine at positions Gly 148 (G148A), Arg 149 (R149A), or Leu151 (L151A) produced by *in vitro* transcription-translation (IVTT) and the amount of these ^{35}S-labeled BAD proteins that were captured by GST-BCL-2 or GST-BCL-X_L bound to GSH-agarose beads in an *in vitro* binding assay, (B) a Western blot of lysates from FL5.12 BCL-X_L cells stably expressing wild-type or mutant forms of BAD probed with an anti-BAD antibody (upper panel) or an anti-BCL-X_L antibody (lower panel), and (C) a western blot analysis of levels of wild-type and mutant BAD proteins in total cell lysates (lysates), in BCL-X_L co-immunoprecipitates from the lysates (IPαBCL-X_L), and in the supernatant following removal of BCL-X_L/BAD complexes (Sup);

Figure 8 illustrates the effects of mutations in BAD BH1 and BH3 domains on intracellular distribution and death promoting activity, showing (A) proteins detected by anti-BAD Ab probing of a Western blot of crude membrane and cytosol fractions from FL5.12BCL-X_L cells expressing WT or mutant BAD proteins, (B) Western blot detection of proteins associated with WT and mutant BAD in the cytosolic fraction as determined by co-immunoprecipitation with anti-BAD mAb 2G11, and (C) a graph of viability of FL5.12BCL-X_L cells expressing WT or mutant BAD proteins as determined by propidium iodine exclusion at 24 hr., 48 hr., and 72 hr. after withdrawal of interleukin-3;

Figure 9 illustrates the effect of BCL-2 BH1, BH2, and BH3 mutations on heterodimerization of BCL-2 with BAD showing ^{35}S-labeled wild-type (WT) and mutant BCL-2 proteins substituted with alanine at positions Gly 145 (G145A), Trp 188 (W188A), or Leu97 (L97A) produced by *in vitro* transcription-translation (IVTT) and the amount of these ^{35}S-labeled BCL-2 proteins that were captured by GST-BAD bound to GSH-agarose beads in an *in vitro* binding assay;

Figure 10 illustrates (A) the BH3 domain of murine BID, represented with two upstream and two downstream amino acids (SEQ ID NOS:19) and a schematic representation of mutations introduced into BID (SEQ ID NOS:20-23) and (B) *in vitro* binding of BCL-2 or BAX with GST-BID or BID mutants;

Figure 11 illustrates (A) the viability of FL5.12-Bcl-2 clones expressing wild type or BH3-domain mutant BID, (B) Western blot showing BID expression and (C) Western blot showing association of wild type or BH3-domain mutant BID with BCL-2 and BAX (Lane 1: FL5.12-Bcl-2/Hygro.1; Lane 2: FL5.12-Bcl-2/Bid-8; Lane 3: FL5.12-Bcl-2/BidmIII-1.15; Lane 4: FL5.12-Bcl-2/BidmIII-2.10;

Lane 5: FL5.12-Bcl-2/BidmIII-3.1; Lane 6: FL5.12-Bcl-2/BidmIII-4.1);

Figure 12 illustrates (A) the viability of Jurkat cells expressing wild type and BH3-domain mutant BID; (B) Western blot showing levels of BID polypeptides; and (C) viability measured in luciferase activity in Rat-1 fibroblasts co-transfected with the luciferase reporter gene and with *bcl-2*, *bcl-2* along with *bid*, and with wild type and BH3-domain mutant *bid*;

Figure 13 illustrates the death-promoting activity of full-length BAX BH3-domain mutants showing (A) the location of substitution mutations made in or near the BH3 domain (SEQ ID NOS:24-29), (B) the luciferase activity in Rat-1 cells co-transfected with a luciferase reporter gene and a recombinant pcDNA3 vector encoding wild-type BAX, a BAX BH3-domain mutant or wild-type BCL-2, and (C) the amount of luciferase activity in Rat-1 cells co-transfected with both BCL-2 and a wild-type or BH3-domain BAX mutant.

Figure 14 illustrates various regions of (A) BAX and (B) BID proteins tested for death-promoting activity when encoded by expression vectors transiently transfected into cells;

Figure 15 illustrates the death-promoting ability of various BAX and BID regions showing (A) and (B) the amount of luciferase expression in Rat-1 cells at 20 hours after co-transfection with or without a pcDNA3 vector encoding BCL-2 and with recombinant pcDNA3 vectors encoding the (A) BAX regions or (B) BID regions, and (C) the amount of luciferase expression in Rat-1 cells grown in the presence or absence of the caspase inhibitor z-VAD-fmk at 20 hrs following transfection with recombinant pcDNA3 vectors encoding the indicated BAX and BID regions;

Figure 16 illustrates the effect of BH3 polypeptides on nuclear morphology of cells showing

photographs of Rat-1 cells transfected with (A) BAX WT, (B) BAX 53-104, (C) BID WT, or (D) BID 74-128 and stained with the DNA dye Hoechest 33342;

Figure 17 illustrates the death-promoting ability of Tat-BH3 peptides showing (A) the sequences of synthetic peptides consisting of an 11 amino acid sequence from the HIV I Tat protein (SEQ ID NO:55) linked to BAX or BID amino acid sequences containing a wild-type or mutant (m) BH3 domain and varying lengths of wild-type flanking region (SEQ ID NOS:30-39) and (B) the viability of 2B4 cells determined by trypan blue dye exclusion at four hours after no treatment or treatment with 100 μM of the Tat peptide or one of the Tat-BH3 peptides shown in (A);

Figure 18 illustrates the kinetics and dose-response relationship of cell death induced by Tat-BH3 peptides containing a wild-type or mutant BH3 domain from BAX or BID showing the viability of 2B4 cells determined by trypan blue dye exclusion (A) at different times following no treatment or treatment with 100 μM of the designated Tat-BH3 peptide and (B) at two hours after treatment with different doses of the Tat-BH3 peptide;

Figure 19 illustrates the effect of BCL-2 and z-VAD-fmk on cell death induced by Tat-BH3 peptides showing (A) the viability of 2B4 cells overexpressing BCL-2 or the vector alone (neo) determined by trypan blue dye exclusion at two hours after no treatment or treatment with Tat-BAX(57-71) or Tat-BID(81-100) at 100 μM concentration in the presence or absence of 200 μM z-VAD-fmk and (B) the percentage of these cells with subdiploid DNA (<2n) as determined by PI staining followed by flow cytometry;

Figure 20 illustrates the effect of Tat-BH3 peptides on cell morphology showing photographs of Jurkat cells treated for two hours with 100 μM of (A, B) Tat-

BAX(57-71) or (C, D) Tat-BID(81-120), stained with the DNA dye Hoescht 33342 and examined by (A, C) phase contrast light microscopy or (B, D) fluorescent microscopy;

Figure 21 illustrates the amino acid sequences for murine and human pro-apoptotic family members showing (A) full-length murine BAD and partial human BAD sequences (SEQ ID NOS:41 and 42), with conservative amino acid substitutions indicated by a dot (.), (B) full-length murine and human BAK sequences (SEQ ID NOS: 43 and 44), (C) full-length murine and human BAX sequences (SEQ ID NOS: 45 and 46), (D) full-length murine and human BID sequences (SEQ ID NOS: 47 and 48), with conservative amino acid substitutions indicated by a dot(.), and (E) full-length human BIK (SEQ ID NO: 49); and

Figure 22 illustrates the nucleotide sequences of human cDNAs showing (A) a partial bad cDNA (SEQ ID NO:50) which encodes a BH3-containing BAD polypeptide, (B) a bak cDNA (SEQ ID NO:51) encoding full-length BAK, (C) a bax cDNA (SEQ ID NO:52) encoding full-length BAX, (D) a bid cDNA (SEQ ID NO:53) encoding full-length BID, and (E) a bik cDNA (SEQ ID NO:54) encoding full-length BIK.

Description of the Preferred Embodiments

The present invention is based, in part, upon the unexpected discovery that BAD, like all other known pro-apoptotic members of the BCL-2 family, has a BH3 domain and that this domain is necessary for BAD's death agonist activity. This discovery was unexpected because BAD has been previously reported as containing only BH1 and BH2 domains in common with BCL-2 family members. Yang et al., *Cell 80*:285-291, 1995, incorporated herein by reference. Moreover, unlike all other BH1- and BH2-containing family members, in which the BH3 domain is located N-terminal to the BH1 domain (Fig. 2), the BH3 domain of BAD is located between the BH1 and BH2 domains

and indeed partially overlaps the C-terminal portion of the BH1 domain (Fig. 2). The heretofore unrecognized presence of a BH3 domain in all known pro-apoptotic members of the BCL-2 family along with the herein described death inducing activity of short BH3-containing polypeptides establishes for the first time that the BH3 domain is sufficient for inducing cell death. It is also believed that peptides as short as five amino acids from the BH3 domain will also have death agonist activity.

Therefore, the present invention provides a BH3 polypeptide of at least 9 and no more than 50 amino acids comprising a BH3 domain of a pro-apoptotic BCL-2 family member. The BH3 domain comprises a nine amino acid sequence as set forth in SEQ ID NO:40: Leu-Xaa_1-Xaa_2-Xaa_3-Xaa_4-Asp-Xaa_5-Xaa_6-Xaa_7, wherein Xaa_1 is Arg or Ala, Xaa_2 is Arg, Ile, Leu, Lys, Gln or Cys, Xaa_3 is Met, Ile or Val, Xaa_4 is Ser or Gly, Xaa_5 is Glu, Asp or Ser, Xaa_6 is Phe, Ile, Leu or Met, and Xaa_7 is Val, Glu, Asn or Asp; or a conservatively substituted variant thereof.

A conservatively substituted variant of SEQ ID NO:40 is an amino acid sequence having identity to or conservative amino acid substitutions at any of the nine positions of SEQ ID NO:42. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids which have neutral and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic side chains (G, A, V, L, and I); another grouping is

those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions are: R-K; E-D, Y-F, L-M; V-I, and Q-H. A conservatively substituted variant of SEQ ID NO:40 also includes the amino acid sequence of a BH3 domain identified in any subsequently discovered BCL-2 family member which has cell death agonist activity.

In preferred embodiments, the BH3 domain is from a mammalian pro-apoptotic BCL-2 family member. More preferably, the BH3 domain is from murine or human BAD, (FIG. 21A) BAK (FIG. 21B), BAX (FIG. 21C), BID (FIG. 21D), or human BIK (FIG. 21E) and comprises an amino acid sequence as set forth in any of SEQ ID NO:1-9 (FIG 1). Most preferably, the BH3 domain is a human amino acid sequence as set forth in any of SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

In addition to the BH3 domain of nine contiguous amino acids, the BH3 polypeptide can comprise at least one and up to 41 additional amino acids which flank the BH3 domain or which are contiguous to the N-terminal or C-terminal amino acids of the BH3 domain. Preferably, the BH3 polypeptide comprises between at least about 9 and about 50 contiguous amino acids and can have a length of any number between 9 and 50. More preferably, the BH3 polypeptide comprises at least 11 amino acids and even more preferably, the BH3 polypeptide is between at least 15 and 24 contiguous amino acids in length.

The amino acid sequence of the BH3 polypeptide can be any sequence provided that it includes a BH3 domain as defined above and that the polypeptide has cell death agonist activity. The term "cell death agonist activity"

is intended to mean that the BH3 polypeptide is capable of inducing cell death in a similar fashion, although not necessarily to the same degree, as the polypeptides particularly exemplified herein. The cell death agonist activity of a polypeptide can be readily examined using one of the cell assays described herein. It is believed that the amino acid sequence of the BH3 polypeptide should be one which folds in such a manner that the BH3 domain is exposed on the surface of the surface of the polypeptide.

Preferably, the BH3 polypeptide comprises a BH3-containing sequence of between at least 9 and 50 contiguous amino acids from a pro-apoptotic BCL-2 family member. Even more preferably, the BH3-containing sequence is from one of the human polypeptide sequences shown in Figure 21: BAD (SEQ ID NO:41), BAK (SEQ ID NO:42), BAX (SEQ ID NO:43), BID (SEQ ID NO:44) or BIK (SEQ ID NO:45), or a conservatively substituted variant thereof. A conservatively substituted variant of a BH3-containing sequence means the sequence contains conservative amino acid substitutions of one or more of the amino acids in the naturally occurring sequence. The BH3 polypeptides of the invention can also include unusual amino acids and/or amino acids containing modifications such as glycosylations.

Preferred BH3 polypeptides are human BAX polypeptides BAX 53-76 (SEQ ID NO:31), BAX 57-71 (SEQ ID NO:33), BAX 61-71 (SEQ ID NO:35), and a human BID polypeptide, BID 81-100 (SEQ ID NO:37), which are defined by reference to the full-length BAX and BID sequences (FIGS. 21C and 21D). Most preferably, the BH3 polypeptide comprises human BAX 57-71 which consists of the sequence Lys-Lys-Leu-Ser-Glu-Cys-Leu-Lys-Arg-Ile-Gly-Asp-Glu-Leu-Asp (SEQ ID NO:33).

The invention also provides BH3 domain peptides having cell death agonist activity. A BH3 domain peptide

comprises five to eight contiguous amino acids from a BH3 domain as defined by SEQ ID NO:40, or a conservatively substituted variant thereof.

Methods for preparation of the BH3 polypeptides and BH3 domain peptides of the invention include, but are not limited to, chemical synthesis, recombinant DNA techniques or isolation from biological samples. Chemical synthesis of a peptide can be performed, for example, by the classical Merrifeld method of solid phase peptide synthesis (Merrifeld, *J Am Chem Soc 85:*2149, 1963 which is incorporated by reference) or the FMOC strategy on a Rapid Automated Multiple Peptide Synthesis system (DuPont Company, Wilmington, DE) (Caprino and Han, *J Org Chem* 37:3404, 1972 which is incorporated by reference).

The polypeptides and peptides of the present invention are also intended to include non-peptidal substances such as peptide mimetics which possess the death-inducing activity of BH3 polypeptides or BH3 domain peptides. The techniques for development of peptide mimetics are well known in the art. (See for example, Navia and Peattie, *Trends Pharm Sci 14*:189-195, 1993; Olson et al, *J Med Chem 36*:3039-3049 which are incorporated by reference). Typically this involves identification and characterization of the interaction between a protein target and its peptide ligand using X-ray crystallography and nuclear magnetic resonance technology. For example, it is believed that at least one target protein for BH3 polypeptides is the hydrophobic cleft formed by the BH1, BH2 and BH3 domains of an anti-apoptotic BCL-2 family member. Using information on a normal peptide-protein complex along with computerized molecular modeling, a pharmacophore hypothesis is developed and analogue compounds are made and tested in an assay system.

In one embodiment, the BH3 polypeptide or BH3 domain peptide is operably linked to a cell penetrating

agent. One such cell penetrating agent is the 11 amino acid Tat peptide of HIV-I (SEQ ID NO:55). The Tat peptide may be directly fused to the BH3 polypeptide or it may contain a short spacer sequence. The cell penetrating agent can also be a conservatively substituted variant of SEQ ID NO:55.

The present invention also includes therapeutic or pharmaceutical compositions comprising the BH3 polypeptide or BH3 domain peptide in an amount effective to promote death. Also encompassed within the present invention are methods for promoting apoptosis in a target cell comprising administering to the cell a death-promoting effective amount of the BH3 polypeptide. The target cell can be treated *ex vivo* or it can be present in a patient.

Such compositions and methods are useful for treating diseases or disease conditions in which the cell death signal is down regulated and the affected cell has an inappropriately diminished propensity for cell death, which is referenced herein as being a decreased apoptotic state. Such diseases include, for example, cancer, other lymphoproliferative conditions, arthritis, inflammation, autoimmune diseases and the like which may result from a down regulation of cell death regulation. The compositions and methods of the invention are also useful in treating diseases or disease conditions in which it is desirable to kill certain types of cells, such as virus-infected or autoantibody-expressing cells.

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including, for example, intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in *ex vivo* treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release

formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF). When it is intended that a BH3 polypeptide be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of the BH3 polypeptide across the blood-brain barrier.

The polypeptide can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, the BH3 polypeptide can be coupled to any substance known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor, and administered by intravenous injection. (See for example, Friden et al., *Science* 259:373-377, 1993 which is incorporated by reference). Furthermore, the BH3 polypeptide can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See for example Davis et al. *Enzyme Eng* 4:169-73, 1978; Burnham, *Am J Hosp Pharm* 51:210-218, 1994 which are incorporated by reference).

Furthermore, the compositions of the invention can also comprise agents which aid in targeting the BH3 polypeptide to a particular cell type and/or delivery into the cytosol of a cell. For example, the BH3 polypeptide can be encapsulated in liposomes that have various targeting ligands on their surface such as monoclonal antibodies that recognize antigens specifically expressed by the target cell or ligands which bind to receptors specific for the target cell. Such methods are well known in the art (see e.g., Amselem et al., *Chem Phys Lipids* 64:219-237, 1993 which is incorporated by reference). The BH3 polypeptide can also

be administered in a capsule comprised of a biocampatible polymer.

For nonparental administration, the compositions can also include absorption enhancers which increase the pore size of the mucosal membrane. Such absorption enhancers, which have been used to enable peptides the size of insulin to be transported across the mucosal membrane, include sodium deoxycholate, sodium glycocholate, dimethyl-β-cyclodextrin, lauroyl-1-lysophosphatidylcholine and other substances having structural similarities to the phospholipid domains of the mucosal membrane.

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. BID can also be incorporated into a solid or semi-solid biologically compatible matrix which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or

penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

It is also contemplated that certain formulations containing the BH3 polypeptide are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations

can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in cell death assays. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

In one embodiment of this invention, a BH3 polypeptide may be therapeutically administered by implanting into patients vectors or cells capable of producing a biologically-active form of the polypeptide or a precursor thereof, i.e. a molecule that can be readily converted to a biologically-active form of the BH3 polypeptide by the body. In one approach, cells transformed to express and secrete the BH3 polypeptide may be encapsulated into semipermeable membranes for implantation into a patient. It is preferred that the cell be of human origin and that the BH3 polypeptide have a human amino acid sequence when the patient is human. However, the formulations and methods herein can be used for veterinary as well as human applications and the term "patient" as used herein is intended to include human and veterinary patients.

Alternatively, the BH3 polypeptide can be administered to a target cell by transfecting the cell with a polynucleotide encoding for expression the BH3 polypeptide. If the target cell is in a patient the encoding polynucleotide can be targeted to the cell using methods known in the art, such as encapsulating the

polynucleotide in liposomes bearing targeting ligands or by non-covalently binding the polynucleotide to a ligand conjugate which directs the polynucleotide to the target cell. See, e.g., Wu et al., U.S. 5,635,383 and WO 95/25809.

The invention also provide polynucleotides encoding the BH3 polypeptides described herein. In particular, the polynucleotide comprises a nucleotide sequence encoding a BH3 domain consisting of the amino acid sequence set forth in SEQ ID NO:40. Preferred polynucleotides comprise a nucleotide sequence from one of the human cDNA sequences shown in Figure 22: bad (SEQ ID NO:47), bax (SEQ ID NO:48), bak (SEQ ID NO:49), bid (SEQ ID NO:50), or bik (SEQ ID NO:51).

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example 1

This example demonstrates that BAD contains a BH3 domain that is required for heterodimerization and cell death.

BAD was initially identified by its interaction with BCL-2 and BCL-X_L. To define the minimal region in BAD essential for its interaction with BCL-2, a nested set of deletion mutants was generated (Fig. 3A) and tested for their ability to interact with BCL-2 protein.

The deletion mutants were prepared by inserting fragments of a murine *bad* cDNA with engineered HindIII and EcoRI sites into the pET17b expression vector in

frame with the T7-gene-10 promoter and the resulting recombinant expression vectors were transformed into BL21 cells (Novagen). One hour after inducing expression of the truncated BAD proteins by IPTG (0.1 mM), total cell lysates were prepared. Lysates (40 µg) were size fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The resulting blot was hybridized with a ^{32}P-labeled glutathione s-transferase - BCL-2 (GST-BCL-2) fusion protein according to the protocol of Blanar and Rutter, *Science 256:*1014-1018, 1992, and the results are shown in Figure 2B.

Each of the BAD proteins 141-181, 141-172, 141-183, and 141-194 exhibited binding to GST-BCL-2 while the truncated BAD proteins 152-204, 163-204, and 173-204 did not bind to GST-BCL-2. Therefore, a small 31-amino acid region (BAD 141-172) is both sufficient and essential for BAD to heterodimerize with BCL-2.

Sequence analysis of this region identified a BAD amino acid sequence (151-159) with homology to BH3 domains found in other pro-apoptotic molecules (Fig. 4). The BH3 domain of BAD is predicted to be an amphipathic α-helix (Fig. 5).

Example 2

This example demonstrates that the BH3 domain is required for BAD's apoptotsis-promoting activity and that BAD deletion mutants lacking the BH3 domain do not bind to BCL-2 or BCL-X_L *in vitro*.

To assess the role of various regions of BAD in promoting apoptosis, full-length and various deletion mutants of BAD were transiently expressed in BAD-deficient murine embryonic fibroblasts (MEF). DNA fragments encoding for full-length BAD or truncated BAD proteins (1-181, 1-141, 127-204, and full-length with a deletion from 142 to 165) (Fig. 6A) and engineered to contain BamHI and EcoRI restriction sites were inserted

into pcDNA3 (Invitrogen), downstream of T7 and CMV promoters. MEF cells were allowed to grow to about 80% confluence in 12-well plates before transfection. A luciferase reporter plasmid (0.1 mg) was mixed with 0.05 mg of a pcDNA3 recombinant construct or the pcDNA3 vector as a control and 3 ml of lipofectAMINE™ (Gibco BRL) and 0.5 ml of the mixture was added to MEF cells for 5 hrs.

The transfected cells were lysed 18-20 hrs later and luciferase assays were performed using a standard substrate (Promega). Luciferase activities were quantified by a luminometer (OptocompII, MGM Instruments Inc.) and the relative luciferase activity for cells co-transfected with a recombinant pcDNA3 construct compared to luciferase activity in cells co-transfected with the control were determined. The means ± ISD of 3 experiments are shown in Fig. 6B.

The effect of recombinantly expressed full-length or truncated BAD on cell viability of the BAD-deficient MEF cells can be estimated by its effect on the activity of the co-transfected luciferase gene, with a low relative luciferase activity indicating low cell viability and high activity indicating good cell viability. As expected, lysates of cells co-transfected with full-length BAD (1-204) showed very little cell viability. In addition, two BAD truncated proteins, BAD 1-181, which was nearly full-length but lacked the BH2 domain, and BAD 127-204, which had a large N-terminal deletion but retained an intact BH1/BH3 region, were nearly as effective as full-length BAD in promoting cell death. In contrast, BAD constructs lacking the BH1/BH3 region (1-141 and Δ142-165) had substantially diminished death-promoting activity.

To assess the effect of this BH1/BH3 region on binding to anti-apoptotic members, an *in vitro* binding assay was performed. Equal amounts of *in vitro* translated, ^{35}S-labeled BCL-2 or BCL-X_L proteins were

incubated with 1 µg of purified GST-BAD fusion protein (wt or mutant) on ice for 30 min. 500 µl of NP-40 buffer with protease inhibitors and 25 µl of GSH-agarose was added to each binding mixture and rotated at 4°C for 1-2 hrs. Materials bound to GSH-agarose were precipitated, washed three times in 1 ml of NP-40 buffer, solubilized in 25 µl of 1X SDS-PAGE sample buffer, and electrophoresed on a 12.5% SDS polyacrylamide gel. An autoradiograph of the gel (not shown) showed that BAD full-length and deletion mutant constructs retaining the BH1/BH3 region formed heterodimers with BCL-2 and BCL-X_L, while BAD deletion mutants lacking the domain failed to bind BCL-2 or BCL-X_L (Fig. 6C). Thus, the BH1/BH3 region (142-165) is required for both heterodimerization and death agonist activity.

Example 3

This example demonstrates that binding of BAD to BCL-2 and BCL-X_L is affected by single amino acid changes in the BAD BH3 domain.

To further dissect the BH1/BH3 region of BAD, BAD mutant proteins were prepared with the following single-amino acid changes: Gly at position 148 to Ala (G148A); Arg at position 149 to Ala (R149A); and Leu at position 151 to Ala (BADL151A). These BAD mutants were generated by site-directed mutagenesis of a murine *bad* cDNA cloned into a pGEM-3Z derivative using the QuikChange site-directed mutagenesis kit (Stratagene). Sequence-confirmed mutant cDNAs and the wild-type murine *bad* cDNA were subcloned into the pSSFV expression vector. The resulting recombinants were used in an *in vitro* transcription-translation system (IVTT, Promega) to generate ^{35}S-labeled wild-type (WT) and mutant BAD proteins, which are shown in the upper panel of FIG. 7A (IVTT).

Binding of the ^{35}S-labeled wild-type and BH1/BH3 mutant BAD proteins to GST-BCL-2 and GST-BCL-X_L fusion proteins was assessed by an *in vitro* binding assay, which was performed as described in Example 2. The amount of radioactively labeled heterodimers captured on GSH agarose beads are shown in the middle and lower panels of FIG. 7A.

Substitutions in the region of BAD homologous to BH1 (G148A and R149A) did not significantly affect the ability of the BAD mutants to bind to BCL-X_L (FIG. 7A, lower panel). However, while binding to BCL-2 was not significantly affected by the R149A mutation, it was reduced approximately 50% by the G148A mutation (middle panel). Of note, replacement of Leu151 of the BH3 domain with alanine (L151A) reduced the binding of mutant BAD with either BCL-2 or BCL-X_L by more than 90%.

Example 4

This example demonstrates the ability of BAD BH1/BH3 mutants to bind to BCL-X_L *in vivo*.

The recombinant pSFFV expression vectors encoding the wild-type BAD and the BAD mutants described in Example 3 were electroporated into the murine hematopoietic cell line FL5.12 BCL-X_L, which overexpresses BCL-X_L. Clones expressing similar levels of WT and mutant BAD proteins as well as BCL-X_L were identified by probing Western blots of cell lysates with either a rabbit polyclonal anti-BAD antibody (#10929, described in Yang et al., *Cell 80:* 285-291, 1995) (Fig. 7B, upper panel) or a rabbit polyclonal anti-BCL-XL antibody (13.6, described in Boise et al., *Immunity 3:* 87-98, 1995) (Fig. 7B, lower panel).

To assess *in vivo* binding, BAD/BCL-X_L heterodimers were immunoprecipitated from cell lysates using 7B2, a murine monoclonal Ab against human BCL-X_L (Boise et al., *supra*). About 5-10 X 10^6 cells were lysed in 100 µl of

NP-40 isotonic lysis buffer with freshly added protease inhibitors (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.2], 1 mM EDTA, 0.25% NP-40, 0.2 mM PMSF, 0.1% aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin), incubated on ice for 30 min, and centrifuged at 15,000 X g for 10 min to precipitate nuclei and non-lysed cells. 20 µg of 7B2 mAb was added to the supernatant of each sample, mixed, and incubated on ice for 30 min. Subsequently 400 µl of NP-40 buffer was added to the sample along with 25 µl of protein A-sepharose and incubated at 4°C with rotation for 1-2 hrs. Immunoprecipitates were collected by a brief spin, washed three times with 1 ml of NP-40 buffer, and solubilized with 1X SDS-PAGE sample buffer. Total cell lysates, immunoprecipitated proteins and the remaining proteins in the BCL-X_L depleted samples were analyzed by western blot for the presence of BAD using the #10929 anti-BAD Ab. The results are shown in FIG. 7C, with the lane labeled IPα BCL-X_L representing the amount of BAD co-immunoprecipitated with BCL-X_L by the 7B2 mAb.

The mutants BAD G148A and BAD R149A were co-precipitated with BCL-X_L in amounts similar to that seen for wild-type BAD (FIG. 7C, compare lanes 2 and 5 with lane 11). However, 7B2 mAb co-precipitated greatly reduced amounts of BAD L151A with BCL-X_L as compared to wild-type BAD (FIG. 7C, compare lanes 8 and 11). Consistent with this, a markedly increased amount of BAD L151A was present in the supernatant (Sup) of this immunoprecipitate compared to the supernatants of the other mutants and wild-type (Sup, compare lane 9 with lanes 3, 6 and 12. This provides *in-vivo* confirmation of the *in vitro* binding results that the L151A mutation in the BH3 domain abolishes binding of BAD to BCL-X_L.

Example 5

This example demonstrates the effect of the BH1/BH3 mutations on intracellular distribution of BAD and apoptotic activity.

BAD is known to exist as a nonphosphorylated form that heterodimerizes with BCL-2 and BCL-X_L at membrane sites and as a hyperphosphorylated form that does not bind to BCL-2 or BCL-X_L but instead binds to the 14-3-3 protein in the cytosol (Zha et al., *supra*). To assess whether the loss of BCL-2 and BCL-X_L binding activity in the BAD L151A mutant corresponded with this intracellular distribution pattern, the inventors compared the intracellular distribution and 14-3-3 binding activity of wild-type BAD and the BH1/BH3 mutants.

The above-described FL5.12 cells co-expressing BCL-X_L and wild-type or mutant BAD proteins were washed with PBS twice, resuspended in Buffer A (10 mM Tris pH 7.5, 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, aprotinin 0.15 U/ml, 20 mM leupeptin, 1 mM PMSF) and incubated on ice for fifteen minutes. Cells were then homogenized in a Dounce homogenizer with fifty strokes and nuclei were removed by centrifugation at 500g for ten minutes. The supernatant was further centrifuged at 315,000g for thirty minutes to separate cytosol from crude membranes. Membrane fractions were solubilized in 1% SDS and centrifuged at 12,000g for five minutes at room temperature. The resulting membrane fractions and cytosol fractions were diluted 1:10 in 1% Triton X-100, 100 mM NaCl in buffer A and analyzed by western blot using the 10929 anti-BAD Ab and the results are shown in FIG. 8A.

The majority of BAD L151A was present in the cytosolic fraction (Cyt), with the more prominent upper band representing the hyperphosphorylated form and the lower band representing the nonphosphorylated form (Fig. 8A, lane 5). In contrast, the majority of wild-type BAD was detected as the nonphosphorylated form in the crude

membrane fraction (CM, lane 8) as was the majority of BAD G148A (lane 2). BAD R149A, which bears a mutation closer to the BH3 domain than G148A, displayed an intracellular distribution pattern that was intermediate between that observed for BAD G148A and L151A.

Binding ability to 14-3-3 was assessed by immunoprecipitation of BAD/14-3-3 complexes from the cytosolic fraction using the anti-BAD mAb 2G11 (Zha et al., *supra*). The amount of 14-3-3 protein in the immunoprecipitates was analyzed by western blot using an anti-14-3-3 antibody from Upstate Biotechnology, Inc., and the results are shown in FIG 8B.

The anti-BAD mAb 2G11 co-precipitated significantly more 14-3-3 protein associated with BAD L151A than with WT BAD or the other mutants. These data indicate that BAD L151A, which is incapable of binding to BCL-X_L, is also functionally inactive and localized to the cytosol where it is bound to 14-3-3.

Since FL5.12 BCL-XL cells expressing wild-type or mutant BAD are dependent upon IL-3 for survival, the viability of these cells was determined by propidium iodine exclusion at 24 hr., 48 hr., and 72 hr. after IL-3 withdrawal to assess the death-promoting ability of the BAD BH1/BH3 mutants. Two independent sets of clones selected for comparable levels of BAD expression were tested and showed similar results. The means ± ISD of triplicate assays are shown in FIG. 8C.

Like wild-type BAD, the mutants BAD G148A and BAD R149A, which have mutations within the BH1-like region, reversed the protective effect of BCL-X_L seen in the BCL-X_L/Hygro control. However, a high percentage of cells expressing BAD L151A were viable compared to the control, indicating this BH3 BAD mutant could no longer promote cell death.

Example 6

This example demonstrates that heterodimer formation between BAD and BCL-2 is destroyed by a single amino acid change in the BCL-2 BH3 domain.

To determine whether the BCL-2 BH3 domain played a role in BCL-2/BAD heterodimerization, three mutant BCL-2 proteins with single amino acid changes in the BH1, BH2 or BH3 domain, G145A, W188A, and L97A, respectively, were generated using site-directed mutagenesis and ^{35}S-labeled by IVTT essentially as described above. The location of the amino acid mutations are referenced with respect to the murine BCL-2 sequence of SEQ ID NO:?. The ability of the BCL-2 mutants to bind to a GST-wild-type BAD fusion protein (GST-BAD) was assessed in an *in vitro* binding assay performed as described above. As shown in FIG. 9, GST-BAD interacted with slightly reduced efficiency to the BCL-2 BH1 mutant (G145A) and weakly to the BH2 mutant (W188A), but not at all to the BCL-2 BH3 mutant (L97A). Thus, BH3 plays a prominent role in heterodimerization for both the death agonist and antagonist.

Example 7

This example illustrates the effect of BH3 domain mutations on the death agonist activity of BID and the binding of BID to BCL-2 or BAX.

The only conserved domain that BID possesses is BH3, prompting a mutational assessment of its functional importance (Figure 10A). BH3-mutant Bid constructs were generated in two steps. First, the 5' portion of the molecule was PCR amplified. The 5' primer added an *Eco*RI site, while the 3' primer ended at the *Nhe*I site 324 bp into the open reading frame. Second, the amplified *Eco*RI/*Nhe*I fragment plus the 3' *Nhe*I/*Eco*RI fragment were ligated into the *Eco*RI site of pBTM. Subsequently, the entire insert was subcloned into pSFFV for transfection into Fl5.12 cells, pcDNA3 for transient transfection,

pUHD10-3 for inducible clones in Jurkat cells and pGEX-HMK for GST-fusion proteins.

The BH3 mutants of BID were tested for their binding to BCL-2 and BAX *in vitro* (Figure 10B). All four mutants tested disrupted BID's interaction with either BCL-2 or BAX. However, the mutants did display different specificities: BIDmIII-1 (M97A,D98A) bound to BAX but not to BCL-2, BIDmIII-3 (G94A) bound to BCL-2 but not BAX, whereas BIDmIII-2 and mIII-4 did not bind to either (Figure 10B).

To determine if this *in vitro* binding data accurately reflected interactions of the BID mutants *in vivo*, we introduced each BID mutant into FL5.12-Bcl-2 cells and selected stable expressing clones. The expression level of BID mutants was comparable to that of a wild-type BID transfectant (Figure 11B). The ability of each mutant to interact with BCL-2 or BAX was assessed by immunoprecipitation with an anti-BID Ab followed by an anti-BCL-2 or anti-BAX immunoblot (Figure 11C). Anti-human-BCL-2 monoclonal Ab 6C8 and biotinylated anti-murine-BAX polyclonal Ab 651 were used for blot analyses (1:2000 and 1:500, respectively). Wild-type BID (lane 2) and BIDmIII-3 (lane 5) interacted with BCL-2 whereas wild-type BID and BIDmIII-1 (lane 3) interacted with BAX *in vivo*, confirming the *in vitro* binding data. BIDmIII-1 was the only mutant which still interacted with BAX, albeit a decreased amount similar to the *in vitro* assay (Figure 11C).

The capacity of BID mutants to counter protection by BCL-2 was assessed in the stably transfected FL5.12-Bcl-2 clones deprived of IL-3 (Figure 11A). Of note, all BH3 mutants of BID were impaired in their capacity to counter protection by BCL-2. Even BIDmIII-3 (G94A) which still avidly heterodimerized with BCL-2 was less effective than wild-type BID. This dissociated the capacity of BID to

form heterodimers with BCL-2 from its reversal of BCL-2 protection (Figure 10A).

This prompted further assessment of the BID mutants in the inducible system in Jurkat cells which does not require another apoptotic signal (Figure 12A). Moreover, Jurkat cells do not express substantial amounts of BCL-2. Despite substantial levels of protein (Figure 12B), BIDmIII-2,-3 & -4 displayed no meaningful death promoting effect (Figure 12A). Only BIDmIII-1 demonstrated substantial killing that was somewhat less than wt BID (Figure 12A), perhaps reflecting its weaker binding to BAX (Figures 10B and 11C). This BID mutant was also analyzed in the transient transfection death assay in Rat-1 fibroblasts. Once again, BIDmIII-1 demonstrated strong killing activity whereas, the activity of BIDmIII-3 & -4 was substantially impaired (Figure 12C). Thus, the BH3 mutations in BID score differently in stable transfectants with high levels of BCL-2 that require an external death stimulus (IL-3 deprivation, Figure 11A); when compared to systems which induce expression of BID and do not require another signal (Figures. 12A and 12C). Of note, the only BID mutant (mIII-1) still active (M97A,D98A) bound BAX but not BCL-2 (Figures 10B and 11C).

Site specific mutagenesis of BID revealed that BH3 was required for death promoting activity. This included the capacity to counter protection by BCL-2 as well as induce a cysteine protease dependent apoptosis when expressed in Jurkat T cells or Rat-1 fibroblasts (Table 1). The central glycine of BH3 was critical to BID's apoptotic activity.

Table 1

	BIDwt	BIDmIII-1	BIDmIII-2	BIDmIII-3	BIDmIII-4
Yeast Two-Hybrid Interactions with BCL-xL	+	-	-	+	-
In Vitro and *In Vivo* BCL-2 Binding	+	-	-	+	-
Counter BCL-2 *FL5.12-Bcl-2	+	-	-	-	-
In Vitro and *In Vivo* BAX Binding	+	+	-	-	-
Death #Jurkat Agonist Activity	+	+	-	-	-
•Rat-1	+	+	ND	-	-

* Ability to counteract BCL-2's death-inhibiting effect in FL5.12-Bcl=2 cells following IL-3 withdrawal;

Ability to induce cell death in Jurkat cells following induction of BID expression by Doxycyclin treatment;

• Transient co-transfection of both Bid and Luciferase plasmids into Rat-1 cells assessed by Luciferase assay.

Instructively, the various BH3 mutants of BID did not score identically in interactions with BCL-2 and BAX or in death agonist assays. BIDmIII-3 (G94A) which binds BCL-2 but not BAX lost its capacity to counter BCL-2 and induce apoptosis. In contrast, BIDmIII-1 (M97A,D98A) still bound BAX but not BCL-2 and retained death agonist activity. Furthermore, the failure of BIDmIII-1 to counter BCL-2 protection dissociates the capacity of BID to reverse BCL-2 protection from its binding to BCL-2. This provides evidence that BID restores apoptosis in

FL5.12-Bcl-2 cells by its death promoting activity that is independent of binding BCL-2 (Table 1).

Example 8

This example illustrates the effect of mutations in the BH3 domain on the dimerizing and death agonist activities of BAX.

Full-length BAX proteins with substitution mutations in or near the BH3 domain were prepared (Fig. 13A) and tested for their dimerization activity using a yeast two-hybrid binding assay. The following results were obtained: (1) all mutants except BAXmIII-1 (L63A, G67A, L70A, M74A) and BAXmIII-2 (L63E) retain the ability to interact with wild-type BAX, which suggests that in homodimers BH3 interacts with another domain(s), probably BH1 or BH2 or both; (2) BAXmIII-4 (G67E) and BAXmIII-5 (M74A) do not interact with BCL-2 and BCL-x_L; and (3) BAXmIII-3 (G67A), had no change in dimerization ability (Table 2).

Table 2

Summary of Bax Mutants in the BH3 Domain

	Yeast Two-Hybrid			*In Vivo* Interactions			Death Agonist Activity	Counteracting Bcl-2
	Bax	Bcl-2	Baxmut	Bax	Bcl-2	Baxmut		
Baxwt	+	+	NA	+	+	NA	+++	+++
mlll-1	-	-	-	-	-	-	+++	+++
mlll-2	-	-	-	-	-	-	+	-
mlll-3	+	+	+	+	+	+	+++	+
mlll-4	+	-	-	+	-	-	++	+
mlll-5	+	-	+	+	+	+	+++	+++

NA, not applicable

To reconfirm the binding specificity of BAX mutants *in vivo*, the polynucleotides encoding these mutants were subcloned into the mammalian expression vector pSFFV and introduced by electroporation into FL5.12 cells over-expressing BCL-2. Clones expressing exogenous HA-tagged mutant BAX were screened by Western blot with a polyclonal anti-BAX Ab 651, and those with the highest amount of expression were retained. Co-immunoprecipitations from ^{35}S-methionine labeled FL5.12-Bcl-2/HA-Bax cells with anti-HA and anti-BCL-2 antibodies confirmed most of the results by yeast two-hybrid system, with one exception: BAXmIII-5 binds to BCL-2 although it does not in yeast (data not shown). Thus the mutants were separated into three groups according to their binding specificity to BAX and BCL-2 in FL5.12 cells: BAXmIII-1 & 2, which do not bind to either; BAXmIII-4, which binds BAX but not BCL-2; and BAXmIII-3 & 5, which bind to both BAX and BCL-2 (Table 2).

To investigate the death-inducing activity of the BAX mutants, a transient transfection system in Rat-1 fibroblasts was used. BAX mutants were subcloned into the mammalian expression vector pcDNA3 under the control of a CMV promoter, and were co-transfected with a luciferase reporter into Rat-1 cells. Luciferase activity assays as described above were performed 16-18 hrs after transfection. Co-transfection of wild-type BAX with the luciferase reporter resulted in a 10-fold decrease in luciferase activity (Fig. 13B) reflecting its apoptosis activity. Mutants 1, 3 and 5 retained close to wild-type activity, while mutants 2 and 4 were 6- and 3-fold less potent then wild-type BAX, respectively (Fig. 13C).

To assess the ability of the BAX mutants to counteract the anit-apoptotic effect of BCL-2, the Rat-1 cells were co-transfected with polynucleotides encoding BCL-2 and wild-type BAX or a BAX mutant. As shown in FIG. 13C, co-transfection of wild-type BAX and BCL-2 resulted in an intermediate luciferase activity confirming the capacity of

BAX to counteract BCL-2. Mutants 1 and 5 retained wild-type like activity, mutant 2 lost 90% of the activity, while mutants 3 and 4 lost 50-60% of the activity.

The fact that BAXmIII-1 acted like wild-type in the functional assays was unexpected because it lost the ability to form dimers with wild-type BAX and BCL-2 based on the yeast two-hybrid and *in vivo* co-IP data. In order to know whether BAXmIII-1 could form homodimers, its ability for self-binding was tested with several assay systems. Results (data not shown) from yeast two-hybrid, *in vitro* binding and co-IP from transiently transfected 293 cells showed that while BAX mutants 3 and 5 form homodimers, BAX mutants 1, 2 and 4 almost completely lost their homodimerization activity.

A comparison of the interaction and cell killing activities of the BH3 mutants (Table 2) suggest that these two properties of BAX are separable. Moreover, the observation that BAXmIII-1 has no dimerizing activity but has death agonist activity suggests that the amphipathic character of the BH3 domain is sufficient for BAX to function as a death promoter.

Example 9

This example demonstrates the death-promoting activity of BAX and BID BH3-containing fragments when expressed in cells.

To assess the role of various regions of BAX and BID in promoting apoptosis, full-length and various deletion mutants (Figure 14A) were transiently expressed in Rat-1 cells with or without co-expression of BCL-2. DNA fragments encoding for full-length or truncated BAX and BAD proteins were engineered to contain BamHI and EcoRI restriction sites and inserted into pcDNA3 (Invitrogen) under the control of the CMV immediate early promoter. The recombinant pcDNA3 constructs, or the pcDN3 vector as a control, were lipo-transfected into Rat-1 cells along with a vector encoding a

luciferase reporter gene essentially as described in Example 2. In separate experiments, a recombinant pcDNA3 encoding BCL-2 was co-transfected. Luciferase activities were measured 20 hrs. after transfection as described above and expressed as the percentage of the control. The data are shown in FIG. 15A and 15B.

All BAX and BID fragments containing the BH3 domain displayed death agonist activity, as indicated by a reduction in luciferase activity compared to the control (FIG. 15A and 15B). Co-expression of BCL-2 countered the death agonist activity of these fragments. In contrast, cells expressing BID 1-73, which lacks the BH3 domain, were as viable as the control (vector, FIG. 15B).

The role of caspase activation in the cell death induced by BAX 53-104 and BID 74-128 was examined by culturing cells expressing these fragments or wild-type BAX or BID in the absence or presence of z-VAD-fmk (50 μM), which is a general caspase inhibitor (FIG. 15C). Although z-VAD-fmk did not significantly inhibit the death of cells expressing BAX wt but did significantly inhibit death of cells expressing BAX 53-104, BID wt, or BID 74-128.

The nuclear morphology of cells expressing BAX 53-104 or BID 74-128 was compared to that of cells expressing the respective full-length molecules by staining the cells with Hoechest 33342, which is a DNA-specific dye (Figure 16).

Example 10

This example demonstrates that small BH3-containing BAX and BID fragments fused to a tat-peptide can promote cell death.

Polypeptides containing an 11 amino acid sequence from the HIV-I Tat 1 protein (SEQ ID NO:48) and a wild-type or mutated BH3 domain (m) of BAX or BID with different lengths of flanking region (FIG. 17A) were chemically synthesized. The amino acid sequence in the mutated BH3 domains are scrambled versions of the sequential order of amino acids in

wild-type BH3 from BAX of BID. It is believed the Tat sequence facilitates entry of the polypeptide into the cells. These Tat-BH3 polypeptides were added to murine T cell hybridoma 2B4 cells at a concentration of 100 μM and cell viability was examined 4 hr. later by trypan blue dye exclusion.

As shown in Figure 17B, treatment of the 2B4 cells with Tat-BAX(53-76) (SEQ ID NO:31), Tat-BAX(57-71) (SEQ ID NO:33), Tat-Bax(61-71) (SEQ ID NO:35) and Tat-BID(81-100) (SEQ ID NO:37) fusion proteins resulted in a greater than 50% reduction in cell viability as determined by trypan blue dye exclusion at 4 hr. compared to viability in control cells with no treatment or treated with the Tat peptide. In contrast, the corresponding polypeptides containing mutated BH3 domains had no death agonist activity [Tat-BAX(53-76)M (SEQ ID NO:32), Tat-BAX(57-71)M (SEQ ID NO:34) and Tat-BID(81-100)M SEQ ID NO:38)]. The failure of Tat-BAX(53-86) and Tat-BID(75-106) to reduce cell viability in this assay is believed to be due to the larger size of these fusion polypeptides, which may inhibit their entry into the cells. Instructively, BAX53-86 displayed cell death agonist activity when expressed by cells (FIG. 15A) and Tat-BID(75-106) reduced viability of 2B4 cells by more than 40% when trypan blue dye exclusion was determined 19 hours after polypeptide addition (data not shown). This data suggests that therapeutic use of polypeptides longer than about 32 amino acids may require that they be administered with additional cell penetrating agents or expressed by polynucleotides transfected into the cell.

Example 11

This example demonstrates cell viability exposed illustrates the kinetics and dose-response relationship of cell death induced by Tat-BH3 polypeptides.

To assess longer term effects on cell death of the Tat-BH3 or Tat-BH3(m) fusion polypeptides, Tat-BAX(53-76), Tat-

BAX(67-71), Tat BID(81-100) or their corresponding BH3 mutant derivatives were added at a concentration of 100 μM to multiple sets of 2B4 cultures and trypan blue dye exclusion was determined at various times after polypeptide addition.

As shown in FIG. 18A, at concentrations of 100 μM, Tat-BID(81-100) achieved its maximum death promoting effect before the Tat-BAX fusion polypeptides, with more than 75% of the 2B4 cells losing viability by 1 hr. after addition of Tat-(BID)81-100 as compared to about 50% or 40% loss of viability in cells treated with Tat-BAX(57-71) or Tat-BAX(53-76), respectively. However, by 16 hours, the greatest reduction in cell viability was displayed by Tat-BAX(57-71), which killed almost all of the cells by that time, with about 15% and 35% of the cells treated with Tat-BID(81-100) and Tat-BAX(53-76) being viable. As expected, the mutant Tat-BH3 fusion polypeptides did not display significant cell killing activity at early times in the assay. Interestingly, one of these, Tat-BAX(57-71)m, reduced cell viability about 35% by 16 hours, indicating the mutant BH3 domain in this polypeptide has a low level of cell death agonist activity.

To assess the potency of these Tat-BH3 fusion polypeptides, Tat-BAX(57-71), Tat-BAX(57-71)m, Tat-BID(81-100), or Tat-BID(81-100)m was added to 2B4 cells at 25, 50, 75, 100, 125, or 150 μM and two hours later cell viability was determined by trypan blue dye exclusion. The results are shown in FIG. 18B.

The dose response curves for Tat-BAX(57-71) and Tat-BID(81-100) were similar, with loss of cell viability increasing with increasing doses of these polypeptides. While the polypeptides were about equally potent at 75 and 100 μM doses, Tat-BAX(57-71) killed a higher percentage of the 2B4 cells at 50 μM than a corresponding dose of Tat-BID(81-100). The Tat fusion polypeptides with mutant BH3

domains displayed no or very little effect on cell viability at all doses tested.

Example 12

This example illustrates that the cell death induced by Tat-BH3 fusion polypeptides is not inhibited by BCL-2 and z-VAD-fmk.

Duplicate cultures of 2B4 cells transfected with a recombinant vector encoding BCL-2 or control cells (neo) were treated with Tat-BAX(57-71) or Tat-BID(81-100) at 100 μM in the presence or absence of 100 μM of z-VAD-fmk. Two hours later, cell viability was measured by trypan blue dye exclusion (FIG. 19A) and the percentage of cells with subdiploid DNA (<2n) was determined by PI staining followed by flow cytometry (FIG. 19B).

In contrast to the cell death induced by BH3-containing fragments expressed in 2B4 cells, the cell death induced by Tat-BH3 polypeptides added to the cells in culture was not significantly reversed by BCL-2, z-VAD-fmk, or when both BCL-2 and z-VAD-fmk were present (FIG. 19A). Also, the percentage of cells with subdiploid DNA was significantly increased in cultures treated with one of the TatBH3 peptides and this increase was not significantly alleviated by z-VAD-fmk (FIG. 19B). Interestingly, the number of Tat-BID treated cells containing subdiploid DNA was reduced somewhat by BCL-2, but no significant reduction was seen for cells treated with Tat-BAX (FIG. 19B).

Example 13

This example demonstrates that cells treated with the Tat-BAX(57-71) or Tat(BID)81-100 polypeptides are morphologically atypical for apoptotic cells.

Jurkat cells were treated for 2 hours with 100 μM of Tat-BAX(57-71) (FIG. 20A, 20B) or Tat(BID)81-100 (FIG. 20C, 20D). The treated cells were stained with Hoechst 33342 and

then examined by phase contrast light microscopy (FIG. 20A, 20C) or fluorescent microscopy (FIG. 20B, 20D).

The light microscope study indicated that cells treated with these peptides had extensive cell membrane changes, including membrane blebbing. The nuclei of these cells, however, did not show the typical morphology seen in apoptosis in that they were not condensed nor fragmented. In most cases, the nuclei remained intact.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: WASHINGTON UNIVERSITY

(ii) TITLE OF INVENTION: CELL DEATH AGONISTS

(iii) NUMBER OF SEQUENCES: 55

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: ST. LOUIS
(D) STATE: MO
(E) COUNTRY: USA
(F) ZIP: 63105

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HENDERSON, MELODIE W
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(C) REFERENCE/DOCKET NUMBER: 6029-6526

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 314-727-5188
(B) TELEFAX: 314-727-6092

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Arg Arg Met Ser Asp Glu Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Arg Arg Met Ser Asp Glu Phe Glu
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Ala Ile Ile Gly Asp Asp Ile Asn
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Ala Leu Ile Gly Asp Asp Ile Asn
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Arg Lys Ile Gly Asp Glu Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Arg Arg Ile Gly Asp Glu Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Ala Gln Val Gly Asp Ser Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Ala Gln Ile Gly Asp Glu Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Ala Cys Ile Gly Asp Glu Met Asp
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Val Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Glu Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Gln Val Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Gln Val Gly Arg Gln Leu Ala Leu Ile Gly Asp Asp Ile Asn Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Lys Lys Leu Ser Glu Cys Leu Arg Lys Ile Gly Asp Glu Leu Asp Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Lys Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser Met Asp Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Ala Leu Ala Leu Arg Leu Ala Cys Ile Gly Asp Glu Met Asp Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Ala Gln Ile Gly Asp Glu Ala Ala His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Ala Gln Ala Ala Ala Ala Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Ala Gln Ile Ala Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Ala Gln Ile Glu Asp Glu Met Asp His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Ser Glu Cys Ala Arg Arg Ile Ala Asp Glu Ala Asp Ser Asn Ala
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Leu Ser Glu Cys Glu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Leu Ser Glu Cys Leu Arg Arg Ile Ala Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Leu Ser Glu Cys Leu Arg Arg Ile Glu Asp Glu Leu Asp Ser Asn Met
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Leu Ser Glu Cys Leu Arg Arg Ile Gly Asp Glu Leu Asp Ser Asn Ala
1 5 10 15

Glu

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp
1 5 10 15

Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile Ala Ala Val Asp
20 25 30

Thr Asp

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp
1 5 10 15

Glu Leu Asp Ser Asn Met Glu Leu
20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Glu Leu Asp Leu Lys Arg
1 5 10 15

Ile Gly Asp Ser Asn Met Glu Leu
20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Lys Lys Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp Glu Leu Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Lys Lys Leu Ser Glu Cys Glu Leu Asp Leu Lys Arg Ile Gly Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Glu Cys Leu Lys Arg Ile Gly Asp Glu Leu Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Asp Ser Glu Ser Gln Glu Glu Ile Ile His Asn Ile Ala Arg His Leu
1 5 10 15

Ala Gln Ile Gly Asp Glu Met Asp His Asn Ile Gln Pro Thr Leu Val
20 25 30

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Glu Ile Ile His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu
1 5 10 15

Met Asp His Asn
20

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Glu Ile Ile His Asn Ile Ala Arg His Gln Ile Gly Asp Glu Met Asp
1 5 10 15

Leu Ala His Asn
20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu Met Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 2
(D) OTHER INFORMATION: /note= "ARGININE OR ALANINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 3
(D) OTHER INFORMATION: /note= "ARGININE, ISOLEUCINE, LEUCINE, LYSINE, GLUTAMIC ACID OR CYSTEINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /note= "METHIONINE, ISOLEUCINE OR VALINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "SERINE OR GLYCINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /note= "GLUTAMIC ACID, ASPARTIC ACID OR SERINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "PHENYLALANINE, ISOLEUCINE, LEUCINE OR METHIONINE"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "VALINE, GLUTAMIC ACID, ASPARAGINE OR ASPARTIC ACID"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Leu Xaa Xaa Xaa Xaa Asp Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 204 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Met Gly Thr Pro Lys Gln Pro Ser Leu Ala Pro Ala His Ala Leu Gly
1               5                   10                  15

Leu Arg Lys Ser Asp Pro Gly Ile Arg Ser Leu Gly Ser Asp Ala Gly
            20                  25                  30

Gly Arg Arg Trp Arg Pro Ala Ala Gln Ser Met Phe Gln Ile Pro Glu
        35                  40                  45

Phe Glu Pro Ser Glu Gln Glu Asp Ala Ser Ala Thr Asp Arg Gly Leu
    50                  55                  60

Gly Pro Ser Leu Thr Glu Asp Gln Pro Gly Pro Tyr Leu Ala Pro Gly
65                  70                  75                  80

Leu Leu Gly Ser Asn Ile His Gln Gln Gly Arg Ala Ala Thr Asn Ser
                85                  90                  95

His His Gly Gly Ala Gly Ala Met Glu Thr Arg Ser Arg His Ser Ser
            100                 105                 110

Tyr Pro Ala Gly Thr Glu Glu Asp Glu Gly Met Glu Glu Glu Leu Ser
        115                 120                 125

Pro Phe Arg Gly Arg Ser Arg Ser Ala Pro Pro Asn Leu Trp Ala Ala
    130                 135                 140

Gln Arg Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Glu Gly
145                 150                 155                 160

Ser Phe Lys Gly Leu Pro Arg Pro Lys Ser Ala Gly Thr Ala Thr Gln
                165                 170                 175

Met Arg Gln Ser Ala Gly Trp Thr Arg Ile Ile Gln Ser Trp Trp Asp
            180                 185                 190

Arg Asn Leu Gly Lys Gly Gly Ser Thr Pro Ser Gln
        195                 200
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Gly Ala Gly Ala Val Glu Ile Arg Ser Arg His Ser Ser Tyr Pro Ala
1               5                   10                  15

Gly Thr Glu Asp Asp Glu Gly Met Gly Glu Glu Pro Ser Pro Phe Arg
            20                  25                  30
```

Gly Arg Ser Arg Ser Ala Pro Pro Asn Leu Trp Ala Ala Gln Arg Tyr
35 40 45

Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Val Asp Ser Phe
50 55 60

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 208 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Lys Val Gly Cys Asp Glu
1 5 10 15

Ser Pro Ser Pro Ser Glu Gln Gln Val Ala Gln Asp Thr Glu Glu Val
20 25 30

Phe Arg Ser Tyr Val Phe Tyr Leu His Gln Gln Glu Gln Glu Thr Gln
35 40 45

Gly Arg Pro Pro Ala Asn Pro Glu Met Asp Asn Leu Pro Leu Glu Pro
50 55 60

Asn Ser Ile Leu Gly Gln Val Gly Arg Gln Leu Ala Leu Ile Gly Asp
65 70 75 80

Asp Ile Asn Arg Arg Tyr Asp Thr Glu Phe Gln Asn Leu Leu Glu Gln
85 90 95

Leu Gln Pro Thr Ala Gly Asn Ala Tyr Glu Leu Phe Thr Lys Ile Ala
100 105 110

Ser Ser Leu Phe Lys Ser Gly Ile Ser Trp Gly Arg Val Val Ala Leu
115 120 125

Leu Gly Phe Gly Tyr Arg Leu Ala Leu Tyr Val Tyr Gln Arg Gly Leu
130 135 140

Thr Gly Phe Leu Gly Gln Val Thr Cys Phe Leu Ala Asp Ile Ile Leu
145 150 155 160

His His Tyr Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly Trp Val Ala
165 170 175

Ala Leu Asn Leu Arg Arg Asp Pro Ile Leu Thr Val Met Val Ile Phe
180 185 190

Gly Val Val Leu Leu Gly Gln Phe Val Val His Arg Phe Phe Arg Ser
195 200 205

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Arg Gln Glu Cys Gly Glu
1               5                  10                  15

Pro Ala Leu Pro Ser Ala Ser Glu Glu Gln Val Ala Gln Asp Thr Glu
            20                  25                  30

Glu Val Phe Arg Ser Tyr Val Phe Tyr Arg His Gln Gln Glu Gln Glu
        35                  40                  45

Ala Glu Gly Val Ala Ala Pro Ala Asp Pro Glu Met Val Thr Leu Pro
    50                  55                  60

Leu Gln Pro Ser Ser Thr Met Gly Gln Val Gly Arg Gln Leu Ala Ile
65                  70                  75                  80

Ile Gly Asp Asp Ile Asn Arg Arg Tyr Asp Ser Glu Phe Gln Thr Met
                85                  90                  95

Leu Gln His Leu Gln Pro Thr Ala Glu Asn Ala Tyr Glu Tyr Phe Thr
            100                 105                 110

Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly Ile Asn Trp Gly Arg Val
        115                 120                 125

Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu Ala Leu His Val Tyr Gln
    130                 135                 140

His Gly Leu Thr Gly Phe Leu Gly Gln Val Thr Arg Phe Val Val Asp
145                 150                 155                 160

Phe Met Leu His His Cys Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly
                165                 170                 175

Trp Val Ala Ala Leu Asn Leu Gly Asn Gly Pro Ile Leu Asn Val Leu
            180                 185                 190

Val Val Leu Gly Val Val Leu Leu Gly Gln Phe Val Val Arg Arg Phe
        195                 200                 205

Phe Lys Ser
    210
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

```
Met Asp Gly Ser Gly Glu Gln Leu Gly Ser Gly Gly Pro Thr Ser Ser
1               5                  10                  15

Glu Gln Ile Met Lys Thr Gly Ala Phe Leu Leu Gln Gly Phe Ile Gln
            20                  25                  30

Asp Arg Ala Gly Arg Met Ala Gly Glu Thr Pro Glu Leu Thr Leu Glu
        35                  40                  45

Gln Pro Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Arg
    50                  55                  60

Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
65                  70                  75                  80

Ala Asp Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ala Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Val Trp Ile Gln Asp Gln Gly Gly Trp Glu Gly
145                 150                 155                 160

Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```
Met Asp Gly Ser Gly Glu Gln Pro Arg Gly Gly Gly Pro Thr Ser Ser
1               5                  10                  15

Glu Gln Ile Met Lys Thr Gly Ala Leu Leu Leu Gln Gly Phe Ile Gln
            20                  25                  30

Asp Arg Ala Gly Arg Met Gly Gly Glu Ala Pro Glu Leu Ala Leu Asp
        35                  40                  45

Pro Val Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys
    50                  55                  60
```

```
Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
65                  70                  75                  80

Ala Ala Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ser Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Gly Trp Ile Gln Asp Gln Gly Gly Trp Asp Gly
145                 150                 155                 160

Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```
Met Asp Ser Glu Val Ser Asn Gly Ser Gly Leu Gly Ala Lys His Ile
1               5                   10                  15

Thr Asp Leu Leu Val Phe Gly Phe Leu Gln Ser Ser Gly Cys Thr Arg
            20                  25                  30

Gln Glu Leu Glu Val Leu Gly Arg Glu Leu Pro Val Gln Ala Tyr Trp
        35                  40                  45

Glu Ala Asp Leu Glu Asp Glu Leu Gln Thr Asp Gly Ser Gln Ala Ser
    50                  55                  60

Arg Ser Phe Asn Gln Gly Arg Ile Glu Pro Asp Ser Glu Ser Gln Glu
65                  70                  75                  80

Glu Ile Ile His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu
                85                  90                  95

Met Asp His Asn Ile Gln Pro Thr Leu Val Arg Gln Leu Ala Ala Gln
            100                 105                 110

Phe Met Asn Gly Ser Leu Ser Glu Glu Asp Lys Arg Asn Cys Leu Ala
        115                 120                 125

Lys Ala Leu Asp Glu Val Lys Thr Ala Phe Pro Arg Asp Met Glu Asn
    130                 135                 140
```

```
Asp Lys Ala Met Leu Ile Met Thr Met Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Ala Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Phe Ser Tyr Val Arg Asn Leu Val Arg Asn
            180                 185                 190

Glu Met Asp
        195
```

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

```
Met Asp Cys Glu Val Asn Asn Gly Ser Ser Leu Arg Asp Glu Cys Ile
1               5                   10                  15

Thr Asn Leu Leu Val Phe Gly Phe Leu Gln Ser Cys Ser Asp Asn Ser
            20                  25                  30

Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
        35                  40                  45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
    50                  55                  60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu
65                  70                  75                  80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
                85                  90                  95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
            100                 105                 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
        115                 120                 125

Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
    130                 135                 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
            180                 185                 190

Gly Met Asp
        195
```

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

```
Met Ser Glu Val Arg Pro Leu Ser Arg Asp Ile Leu Met Glu Thr Leu
1               5                  10                  15

Leu Tyr Glu Gln Leu Leu Glu Pro Pro Thr Met Glu Val Leu Gly Met
            20                  25                  30

Thr Asp Ser Glu Glu Asp Leu Asp Pro Met Glu Asp Phe Asp Ser Leu
        35                  40                  45

Glu Cys Met Glu Gly Ser Asp Ala Leu Ala Leu Arg Leu Ala Cys Ile
    50                  55                  60

Gly Asp Glu Met Asp Val Ser Leu Arg Ala Pro Arg Leu Ala Gln Leu
65                  70                  75                  80

Ser Glu Val Ala Met His Ser Leu Gly Leu Ala Phe Ile Tyr Asp Gln
                85                  90                  95

Thr Glu Asp Ile Arg Asp Val Leu Arg Ser Phe Met Asp Gly Phe Thr
            100                 105                 110

Thr Leu Lys Glu Asn Ile Met Arg Phe Trp Arg Ser Pro Asn Pro Gly
        115                 120                 125

Ser Trp Val Ser Cys Glu Gln Val Leu Leu Ala Leu Leu Leu Leu Leu
    130                 135                 140

Ala Leu Leu Leu Pro Leu Leu Ser Gly Gly Leu His Leu Leu Leu Lys
145                 150                 155                 160
```

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 190 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

```
GGCGCTGGGG CTGTGGAGAT CCGGAGTCGC CACAGCTCCT ACCCCGCGGG GACGGAGGAC      60

GACGAAGGGA TGGGGGAGGA GCCCAGCCCC TTTCGGGGCC GCTCGCGCTC GGCGCCCCCC     120
```

AACCTCTGGG CAGCACAGCG CTATGGCCGC GAGCTCCGGA GGATGAGTGA CGAGTTTGTG 180

GACTCCTTTA 190

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2094 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GAGGATCTAC AGGGGACAAG TAAAGGCTAC ATCCAGATGC CGGGAATGCA CTGACGCCCA 60

TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC TGGGAGCAGC AGCCGCCAGC CCCTCGGACC 120

TCCATCTCCA CCCTGCTGAG CCACCCGGGT TGGGCCAGGA TCCCGGCAGG CTGATCCCGT 180

CCTCCACTGA GACCTGAAAA ATGGCTTCGG GGCAAGGCCC AGGTCCTCCC AGGCAGGAGT 240

GCGGAGAGCC TGCCCTGCCC TCTGCTTCTG AGGAGCAGGT AGCCCAGGAC ACAGAGGAGG 300

TTTTCCGCAG CTACGTTTTT TACCGCCATC AGCAGGAACA GGAGGCTGAA GGGGTGGCTG 360

CCCCTGCCGA CCCAGAGATG GTCACCTTAC CTCTGCAACC TAGCAGCACC ATGGGGCAGG 420

TGGGACGGCA GCTCGCCATC ATCGGGGACG ACATCAACCG ACGCTATGAC TCAGAGTTCC 480

AGACCATGTT GCAGCACCTG CAGCCCACGG CAGAGAATGC CTATGAGTAC TTCACCAAGA 540

TTGCCACCAG CCTGTTTGAG AGTGGCATCA ATTGGGGCCG TGTGGTGGCT CTTCTGGGCT 600

TCGGCTACCG TCTGGCCCTA CACGTCTACC AGCATGGCCT GACTGGCTTC CTAGGCCAGG 660

TGACCCGCTT CGTGGTCGAC TTCATGCTGC ATCACTGCAT TGCCCGGTGG ATTGCACAGA 720

GGGGTGGCTG GGTGGCAGCC CTGAACTTGG GCAATGGTCC CATCCTGAAC GTGCTGGTGG 780

TTCTGGGTGT GGTTCTGTTG GGCCAGTTTG TGGTACGAAG ATTCTTCAAA TCATGACTCC 840

CAAGGGTGCC CTTTGGGTCC CGGTTCAGAC CCCTGCCTGG ACTTAAGCGA AGTCTTTGCC 900

TTCTCTGTTC CCTTGCAGGG TCCCCCCTCA AGAGTACAGA AGCTTTAGCA AGTGTGCACT 960

CCAGCTTCGG AGGCCCTGCG TGGGGGCCAG TCAGGCTGCA GAGGCACCTC AACATTGCAT 1020

GGTGCTAGTG CCCTCTCTCT GGGCCCAGGG CTGTGGCCGT CTCCTCCCTC AGCTCTCTGG 1080

GACCTCCTTA GCCCTGTCTG CTAGGCGCTG GGGAGACTGA TAACTTGGGG AGGCAAGAGA 1140

CTGGGAGCCA CTTCTCCCCA GAAAGTGTTT AACGGTTTTA GCTTTTTATA ATACCCTTGT 1200

GAGAGCCCAT TCCCACCATT CTACCTGAGG CCAGGACGTC TGGGGTGTGG GGATTGGTGG 1260

GTCTATGTTC CCCAGGATTC AGCTATTCTG GAAGATCAGC ACCCTAAGAG ATGGGACTAG 1320

GACCTGAGCC TGGTCCTGGC CGTCCCTAAG CATGTGTCCC AGGAGCAGGA CCTACTAGGA 1380

GAGGGGGGCC AAGGTCCTGC TCAACTCTAC CCCTGCTCCC ATTCCTCCCT CCGGCCATAC 1440

TGCCTTTGCA GTTGGACTCT CAGGGATTCT GGGCTTGGGG TGTGGGGTGG GGTGGAGTCG 1500

CAGACCAGAG CTGTCTGAAC TCACGTGTCA GAAGCCTCCA AGCCTGCCTC CCAAGGTCCT 1560

CTCAGTTCTC TCCCTTCCTC TCTCCTTATA GACACTTGCT CCCAACCCAT TCACTACAGG 1620

TGAAGGCTCT CACCCATCCC TGGGGGCCTT GGGTGAGTGG CCTGCTAAGG CTCCTCCTTG 1680

CCCAGACTAC AGGGCTTAGG ACTTGGTTTG TTATATCAGG GAAAAGGAGT AGGGAGTTCA 1740

TCTGGAGGGT TCTAAGTGGG AGAAGGACTA TCAACACCAC TAGGAATCCC AGAGGTGGAT 1800

CCTCCCTCAT GGCTCTGGCA CAGTGTAATC CAGGGGTGTA GATGGGGGAA CTGTGAATAC 1860

TTGAACTCTG TTCCCCCACC CTCCATGCTC CTCACCTGTC TAGGTCTCCT CAGGGTGGGG 1920

GGTGACAGTG CCTTCTCTAT TGGCACAGCC TAGGGTCTTG GGGGTCAGGG GGGAGAAGTT 1980

CTTGATTCAG CCAAATGCAG GGAGGGGAGG CAGATGGAGC CCATAGGCCA CCCCCTATCC 2040

TCTGAGTGTT TGGAAATAAA CTGTGCAATC CCCTCAAAAA AAAAACGGAG ATCC 2094

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 579 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

ATGGACGGGT CCGGGGAGCA GCCCAGAGGC GGGGGGCCCA CCAGCTCTGA GCAGATCATG 60

AAGACAGGGG CCCTTTTGCT TCAGGGTTTC ATCCAGGATC GAGCAGGGCG AATGGGGGGG 120

GAGGCACCCG AGCTGGCCCT GGACCCGGTG CCTCAGGATG CGTCCACCAA GAAGCTGAGC 180

GAGTGTCTCA AGCGCATCGG GGACGAACTG GACAGTAACA TGGAGCTGCA GAGGATGATT 240

GCCGCCGTGG ACACAGACTC CCCCCGAGAG GTCTTTTTCC GAGTGGCAGC TGACATGTTT 300

TCTGACGGCA ACTTCAACTG GGGCCGGGTT GTCGCCCTTT TCTACTTTGC CAGCAAACTG 360

GTGCTCAAGG CCCTGTGCAC CAAGGTGCCG GAACTGATCA GAACCATCAT GGGCTGGACA 420

TTGGACTTCC TCCGGGAGCG GCTGTTGGGC TGGATCCAAG ACCAGGGTGG TTGGGACGGC 480

CTCCTCTCCT ACTTTGGGAC GCCCACGTGG CAGACCGTGA CCATCTTTGT GGCGGGAGTG 540

CTCACCGCCT CGCTCACCAT CTGGAAGAAG ATGGGCTGA 579

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 588 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

ATGGACTGTG AGGTCAACAA CGGTTCCAGC CTCAGGGATG AGTGCATCAC AAACCTACTG 60

GTGTTTGGCT TCCTCCAAAG CTGTTCTGAC AACAGCTTCC GCAGAGAGCT GGACGCACTG 120

GGCCACGAGC TGCCAGTGCT GGCTCCCCAG TGGGAGGGCT ACGATGAGCT GCAGACTGAT 180

GGCAACCGCA GCAGCCACTC CCGCTTGGGA AGAATAGAGG CAGATTCTGA AAGTCAAGAA 240

GACATCATCC GGAATATTGC CAGGCACCTC GCCCAGGTCG GGGACAGCAT GGACCGTAGC 300

ATCCCTCCGG GCCTGGTGAA CGGCCTGGCC CTGCAGCTCA GGAACACCAG CCGGTCGGAG 360

GAGGACCGGA ACAGGGACCT GGCCACTGCC CTGGAGCAGC TGCTGCAGGC CTACCCTAGA 420

GACATGGAGA AGGAGAAGAC CATGCTGGTG CTGGCCCTGC TGCTGGCCAA GAAGGTGGCC 480

AGTCACACGC CGTCCTTGGC TCCGTGATGT CTTTCACACA ACAGTAATTT TATTAACCAG 540

AACCTACGCA CCTACGTGAG GAGCTTAGCC AGAAATGGGA TGGACTGA 588

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 923 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CAGCATCGCC GCCGCCAGAG GAGAAATGTC TGAAGTAAGA CCCCTCTCCA GAGACATCTT 60

GATGGAGACC CTCCTGTATG AGCAGCTCCT GGAACCCCCG ACCATGGAGG TTCTTGGCAT 120

GACTGACTCT GAAGAGGACC TGGACCCTAT GGAGGACTTC GATTCTTTGG AATGCATGGA 180

GGGCAGTGAC GCATTGGCCC TGCGGCTGGC CTGCATCGGG GACGAGATGG ACGTGAGCCT 240

CAGGGCCCCG CGCCTGGCCC AGCTCTCCGA GGTGGCCATG CACAGCCTGG GTCTGGCTTT 300

CATCTACGAC CAGACTGAGG ACATCAGGGA TGTTCTTAGA AGTTTCATGG ACGGTTTCAC 360

CACACTTAAG GAGAACATAA TGAGGTTCTG GAGATCCCCG AACCCCGGGT CCTGGGTGTC 420

CTGCGAACAG GTGCTGCTGG CGCTGCTGCT GCTGCTGGCG CTGCTGCTGC CGCTGCTCAG 480

CGGGGGCCTG CACCTGCTGC TCAAGTGAGC CCCCGGCGGC TCAGGCGTGG CTGGCCCCAC 540

CCCCATGACC ACTGCCCTGA GGTGGCGGCC TGCTGCTGTT ATCTTTTTAA CTGTTTTCTC 600

ATGATGCCTT TTATATTAAC CCCGTGATAG TGCTGGAACA CTGCTGAGGT TTTATACTCA 660

GGTTTTTTGT TTTTTTTTTA TTCCAGTTTT CGTTTTTTCT AAAAGATGAA TTCCTATGGC 720

TCTGCAATTG TCACCGGTTA ACTGTGGCCT GTGCCCAGGA AGAGCCATTC ACTCCTGCCC 780

CTGCCCACAC GGCAGGTAGC AGGGGGAGTG CTGGTCACAC CCCTGTGTGA TATGTGATGC 840

CCTCGGCAAA GAATCTACTG GAATAGATTC CGAGGAGCAG GAGTGCTCAA TAAAATGTTG 900

GTTTCCAGCA AAAAAAAAAA AAA 923

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg
1 5 10

What is Claimed is:

1. A bcl-homology domain 3 polypeptide (BH3 polypeptide) comprising a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) the BH3 polypeptide consists of no more than 50 contiguous amino acids, and

(c) the BH3 polypeptide has cell death agonist activity.

2. The BH3 polypeptide of claim 1, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a conservative substituted variant thereof.

3. The BH3 polypeptide of claim 1, which comprises 15 to 24 contiguous amino acids.

4. The BH3 polypeptide of claim 1, which comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

5. The BH3 polypeptide of claim 1, which comprises a human BID polypeptide consisting of SEQ ID NO:37.

6. The BH3 polypeptide of claim 1 which is operably linked to a cell penetrating agent.

7. The BH3 polypeptide of claim 7, wherein the cell-penetrating agent is a Tat peptide as set forth in SEQ ID NO:55 or a conservatively substituted thereof.

8. A polynucleotide encoding a BH3 polypeptide which comprises a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) the BH3 polypeptide consists of no more than 50 contiguous amino acids, and

(c) the BH3 polypeptide has cell death agonist activity.

9. The polynucleotide of claim 8, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9 or a conservative substituted variant thereof.

10. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises 15 to 24 contiguous amino acids.

11. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

12. The polynucleotide of claim 8, wherein the BH3 polypeptide comprises a human BID polypeptide consisting of SEQ ID NO:37.

13. A method for promoting apoptosis in a target cell comprising administering to the cell a death-promoting effective amount of a BH3 polypeptide which comprises a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family,

(b) consists of no more than 50 contiguous amino acids, and

(c) has cell death agonist activity.

14. The method of claim 13, wherein the target cell is present in a human patient and is a cancer cell, a virus-infected cell, or an auto-antibody-producing cell.

15. The method of claim 14, wherein the BH3 domain is a human amino acid sequence as set forth in SEQ ID NO:1, SEQ IN NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.

16. The method of claim 14, wherein the BH3 polypeptide comprises 15 to 24 contiguous amino acids.

17. The method of claim 14, wherein the BH3 polypeptide comprises a human BAX polypeptide consisting of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:35.

18. The method of claim 14, wherein the BH3 polypeptide comprises a human BID fragment consisting of SEQ ID NO:37.

19. The method of claim 14, wherein the BH3 polypeptide is operably linked to a cell penetrating agent.

20. The method of claim 14, wherein the administering step comprises transfecting the cell with a polynucleotide encoding for expression the BH3 polypeptide.

21. A bcl-homology domain 3 peptide (BH3 domain peptide) comprising five to eight amino acids from a BH3 domain as set forth in SEQ ID NO:40, or a conservatively substituted variant thereof, wherein

(a) the BH3 domain is derived from a pro-apoptotic member of the BCL-2 family, and

(b) the BH3 domain peptide has cell death agonist activity.

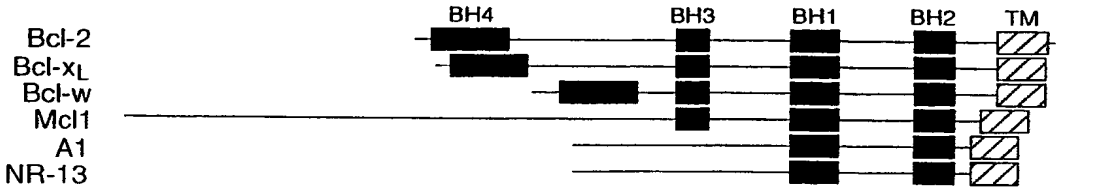
hBAD		L	R	R	M	S	D	E	F	V		SEQ ID NO:1
mBAD	151	L	R	R	M	S	D	E	F	E	159	SEQ ID NO:2
hBAK	78	L	A	I	I	G	D	D	I	N	86	SEQ ID NO:3
mBAK	75	L	A	L	I	G	D	D	I	N	83	SEQ ID NO:4
hBAX	63	L	R	K	I	G	D	E	L	D	71	SEQ ID NO:5
mBAX	63	L	R	R	I	G	D	E	L	D	71	SEQ ID NO:6
hBID	90	L	A	Q	V	G	D	S	M	D	98	SEQ ID NO:7
mBID	90	L	A	Q	I	G	D	E	M	D	98	SEQ ID NO:8
hBIK	61	L	A	C	I	G	D	E	M	D	69	SEQ ID NO:9

Figure 1

THE BCL-2 FAMILY

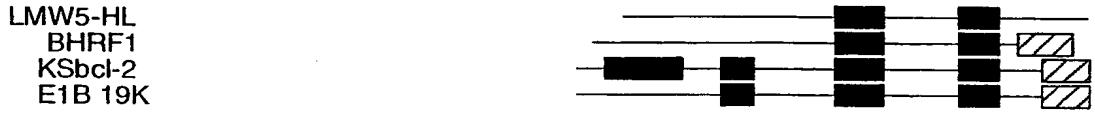
ANTI-APOPTOTIC

MAMMALIAN

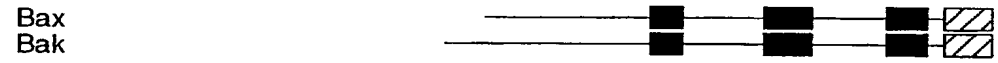


C. elegans

VIRAL HOMOLOGS



PRO-APOPTOTIC



PRO-APOPTOTIC — BH3

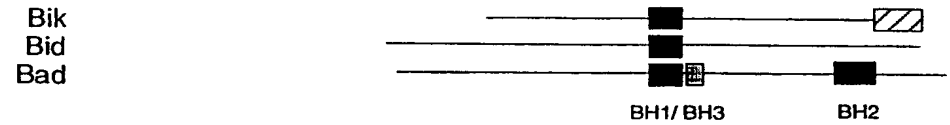


FIGURE 2

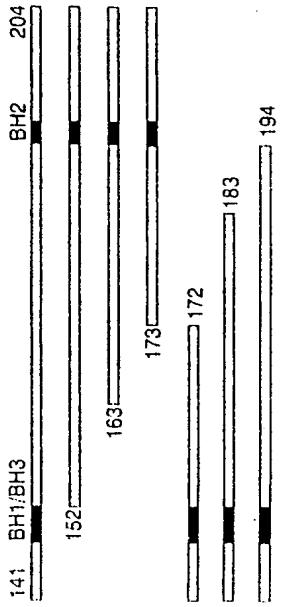


Figure 3A

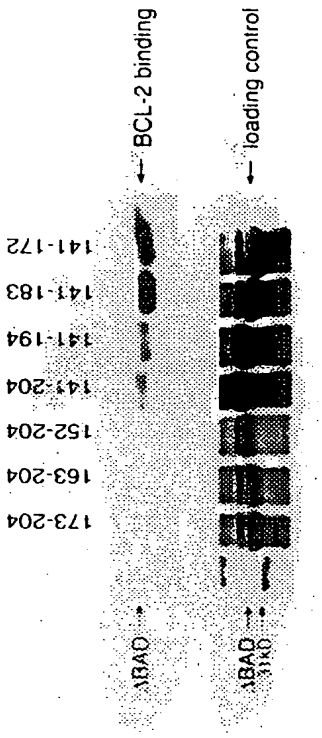


Figure 3B

hBAD		Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	V	D	
mBAD	145	Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	E	G	160
Hbak	72	G	Q	V	G	R	Q	L	A	I	I	G	D	D	I	N	R	87
mBAK	69	G	Q	V	G	R	Q	L	A	L	I	G	D	D	I	N	R	84
hBAX	57	K	K	L	S	E	C	L	R	K	I	G	D	E	L	D	S	72
mBAX	57	K	K	L	S	E	C	L	R	R	I	G	D	E	L	D	S	72
hBID	84	R	N	I	A	R	H	L	A	Q	V	G	D	S	M	D	R	99
mBID	84	H	N	I	A	R	H	L	A	Q	I	G	D	E	M	D	H	99
hBIK	55	D	A	L	A	L	R	L	A	C	I	G	D	E	M	D	V	70

BH3 Domain

SEQ ID NO:10
SEQ ID NO:11
SEQ ID NO:12
SEQ ID NO:13
SEQ ID NO:14
SEQ ID NO:15
SEQ ID NO:16
SEQ ID NO:17
SEQ ID NO:18

Figure 4

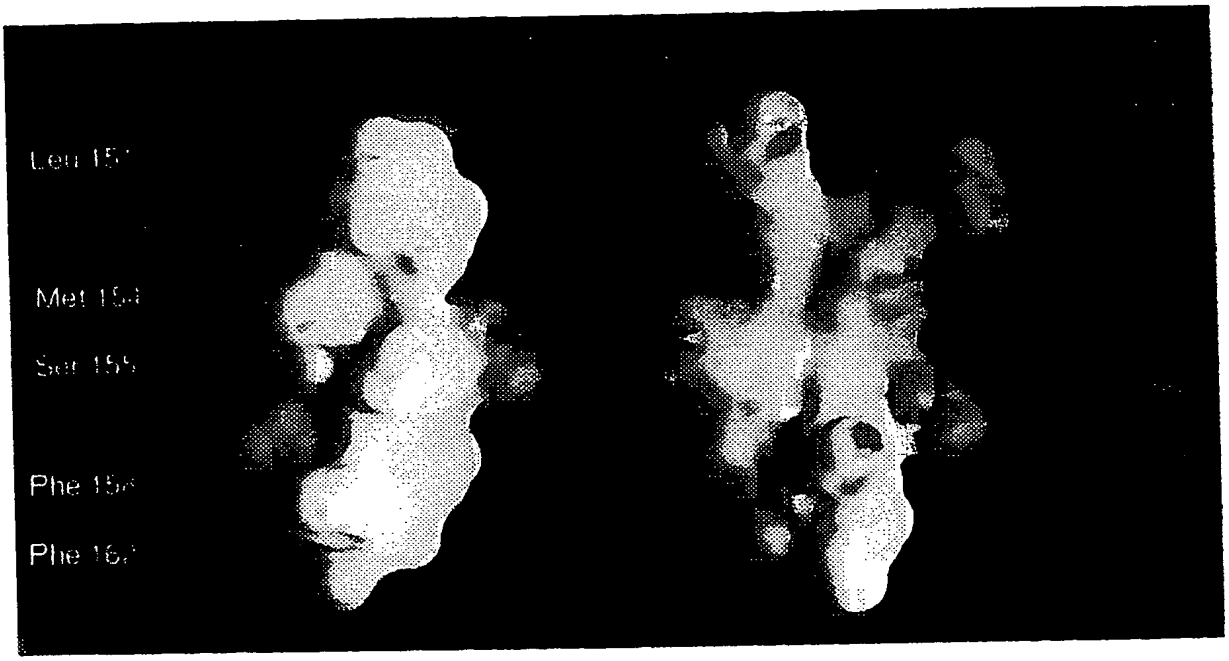


Figure 5

Figure 6A

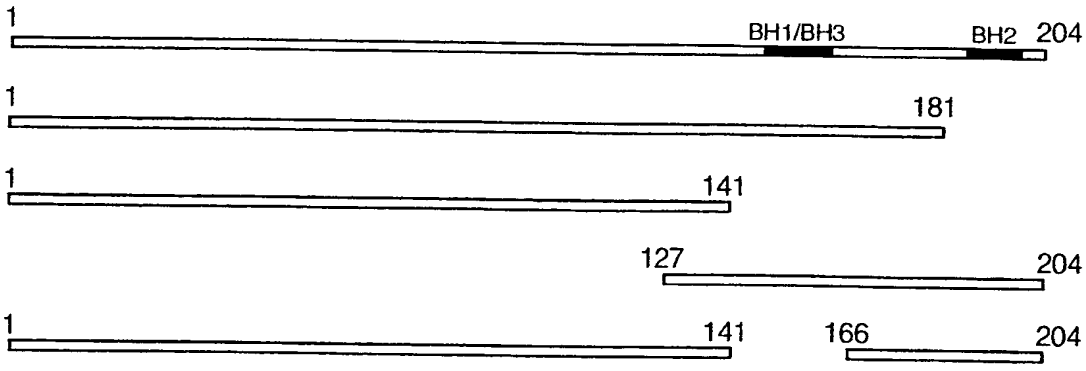


Figure 6B

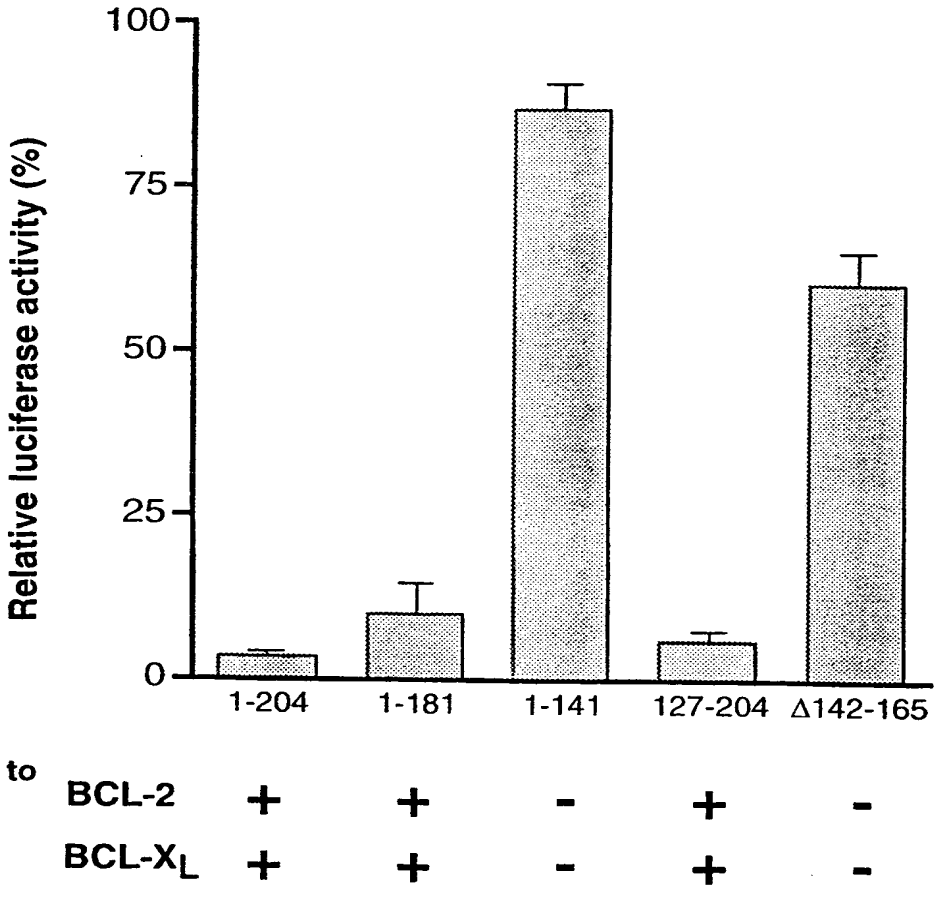


Figure 6C

Figure 7A

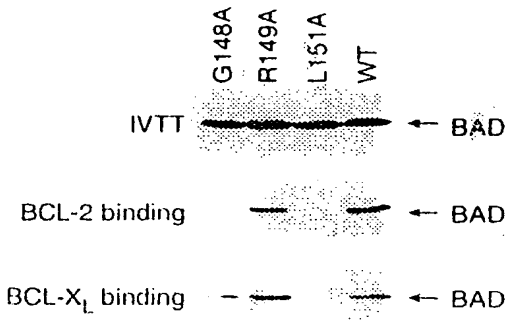
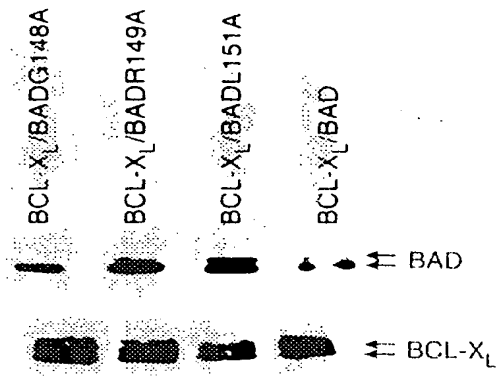


Figure 7B



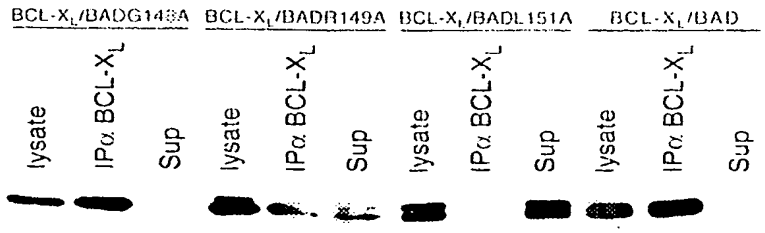


Figure 7C

Figure 8A

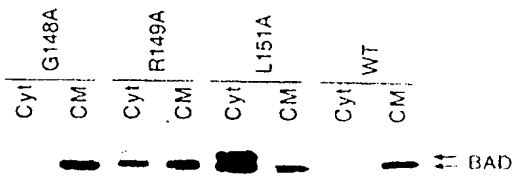


Figure 8B

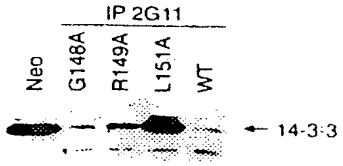
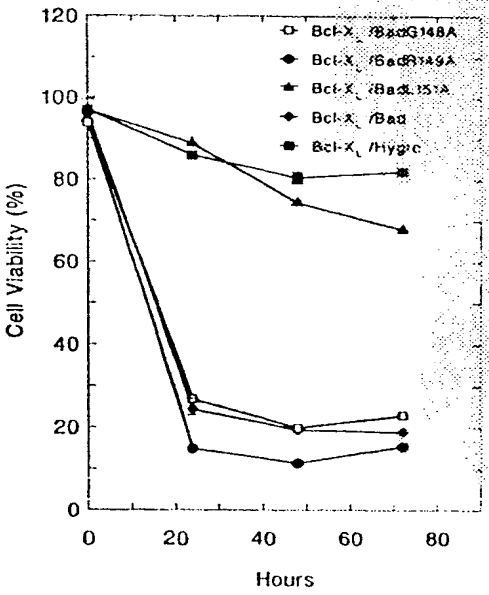


Figure 8C



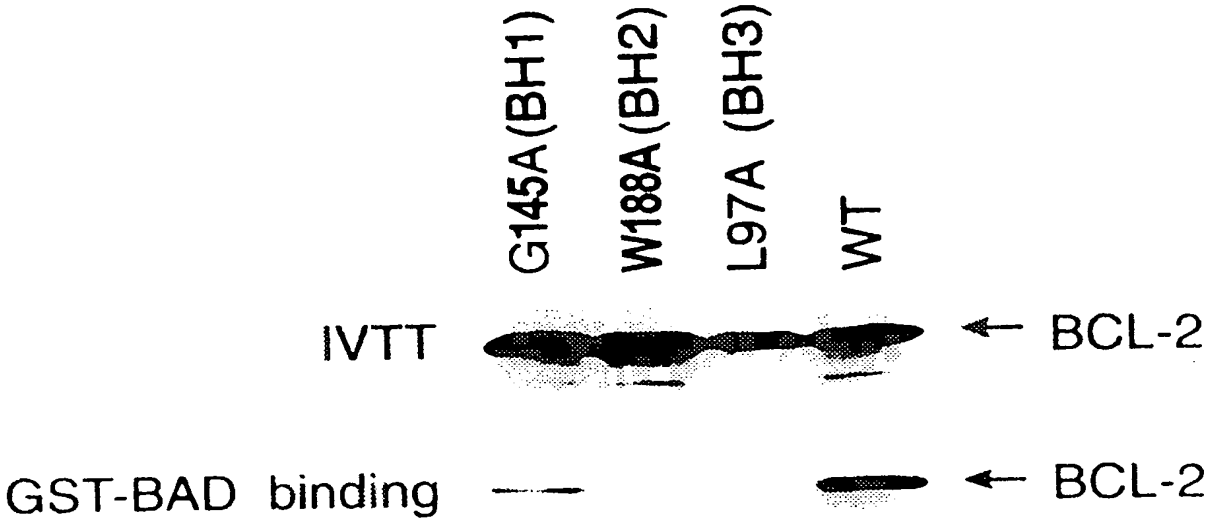


Figure 9

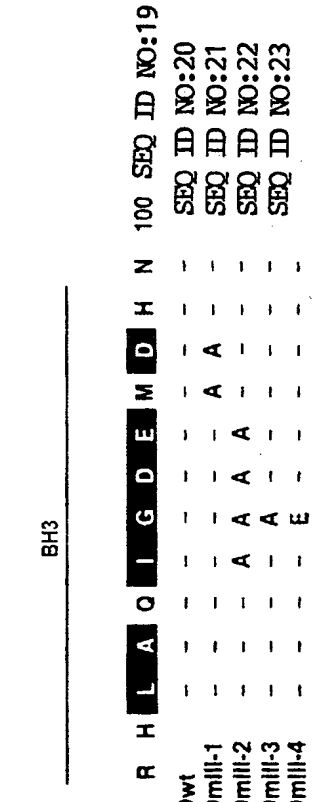


Figure 10A

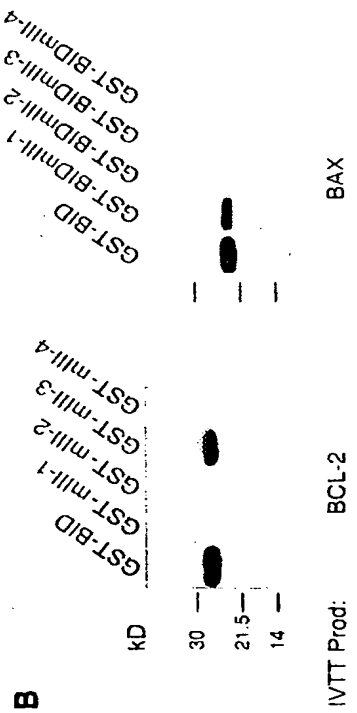


Figure 10B

Figure 11A

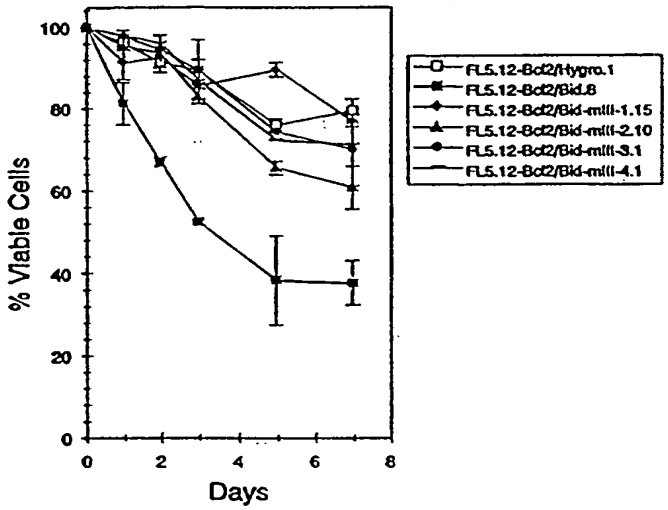


Figure 11B

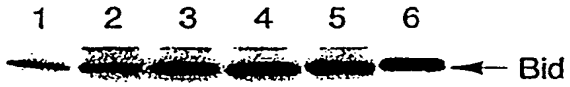


Figure 11C

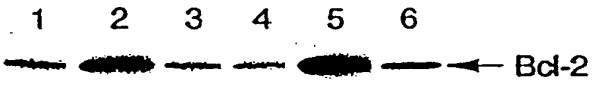


Figure 12B

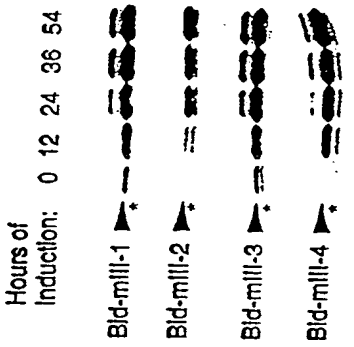


Figure 12A

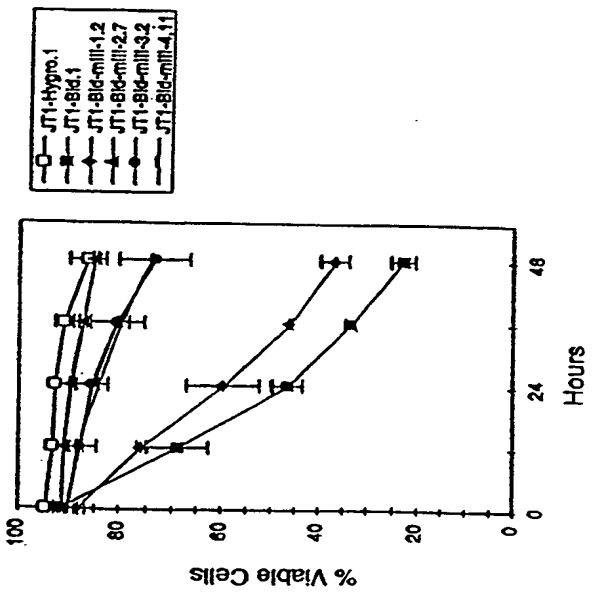
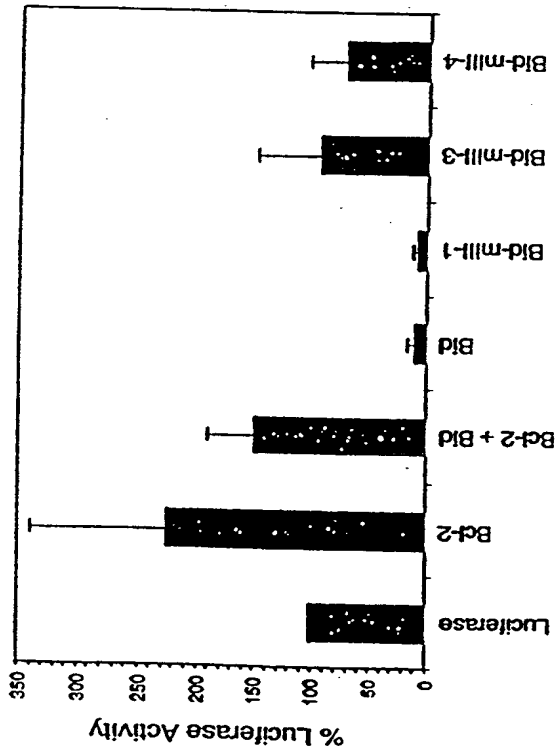


Figure 12C



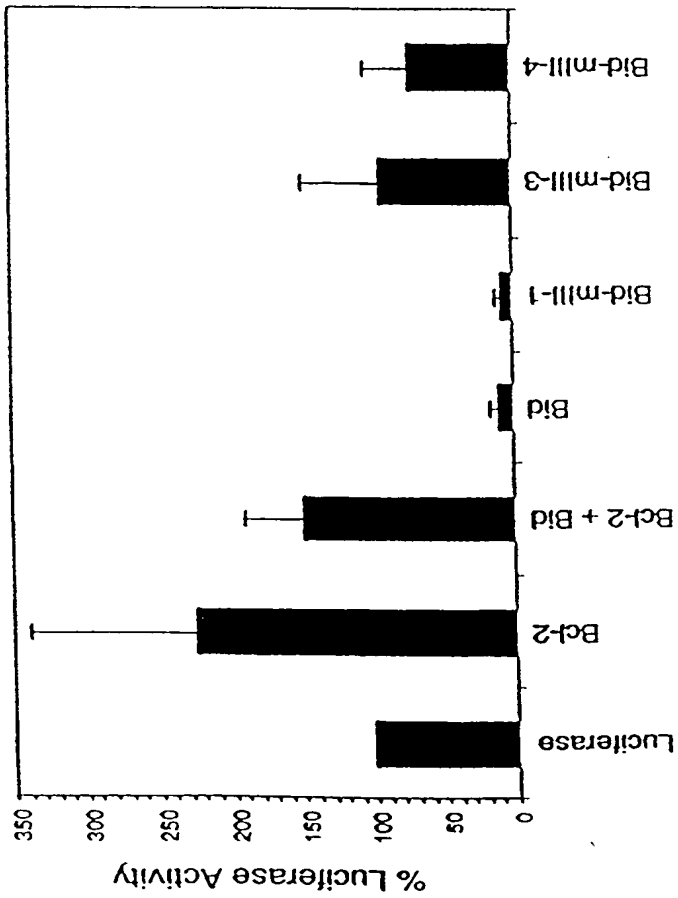


Figure 12C

						BH3														
mBAX	59	L	S	E	C	L	R	R	I	G	D	E	L	D	S	N	M	E	75	SEQ ID NO:24
mll-1		-	-	-	-	A	-	-	-	A	-	-	A	-	-	-	A	-		SEQ ID NO:25
mll-2		-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-		SEQ ID NO:26
mll-3		-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-		SEQ ID NO:27
mll-4		-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-		SEQ ID NO:28
mll-5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-		SEQ ID NO:29

Figure 13A

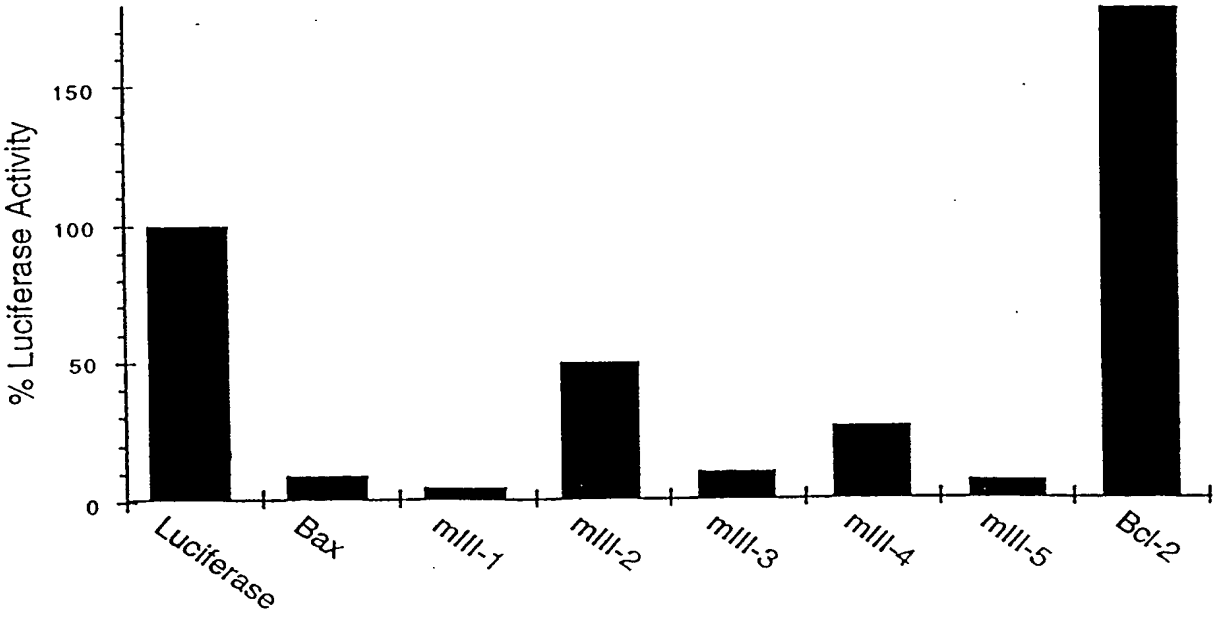


Figure 13B

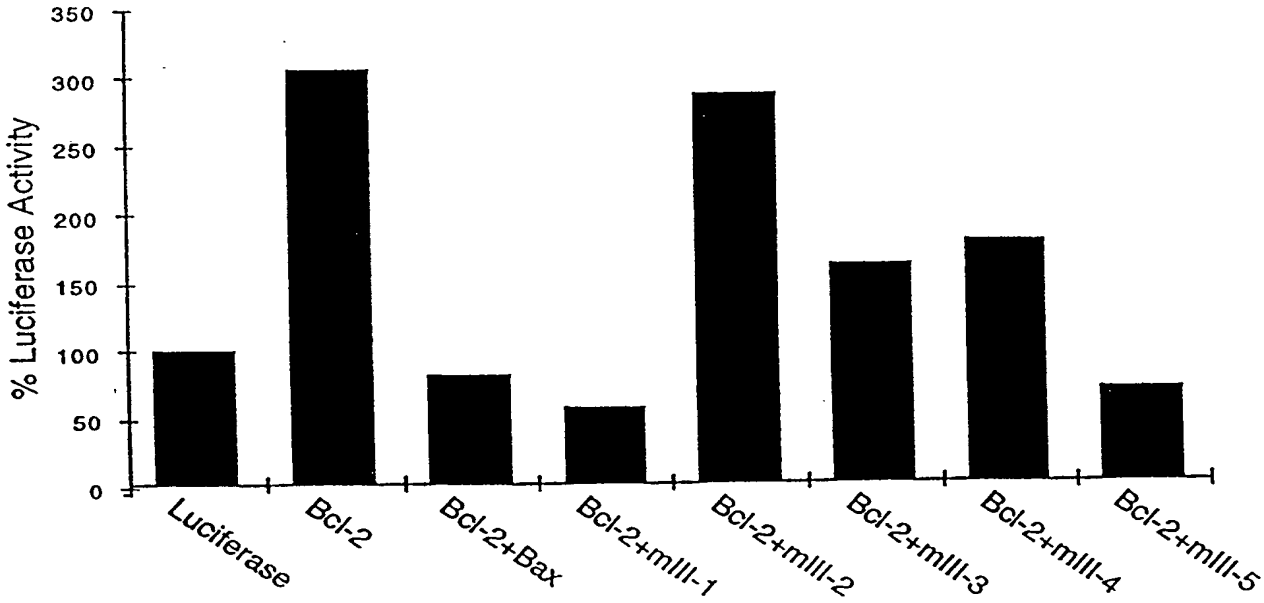


Figure 13C

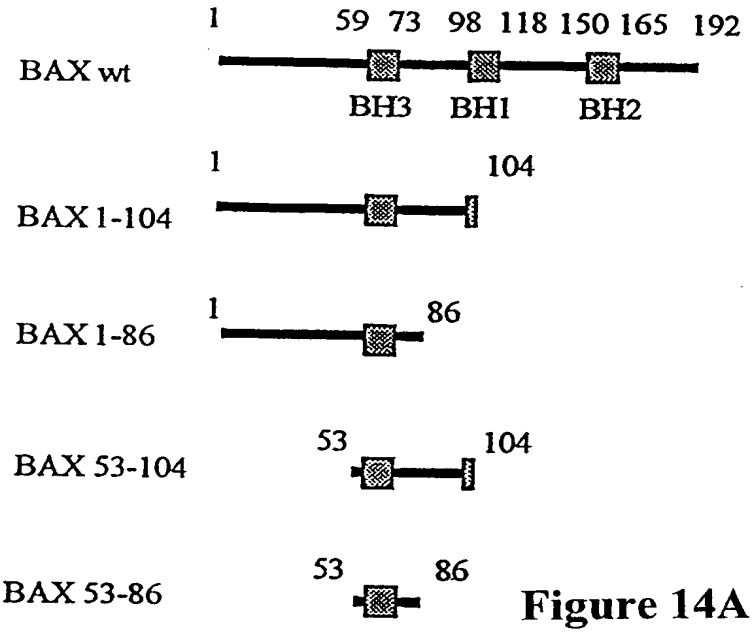


Figure 14A

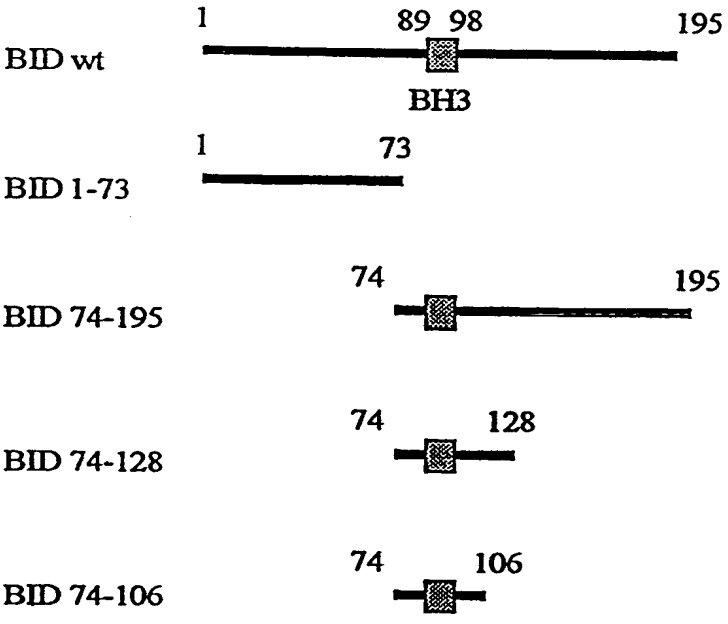


Figure 14B

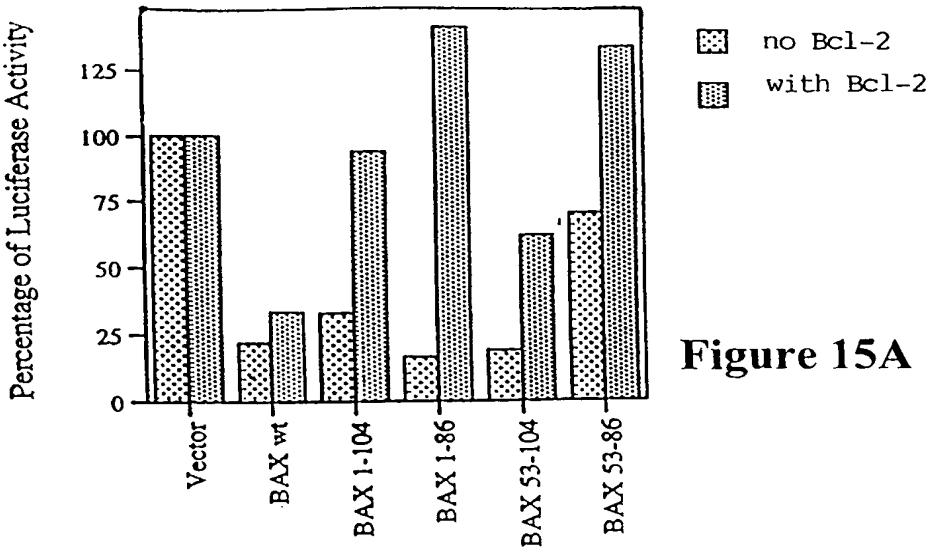


Figure 15A

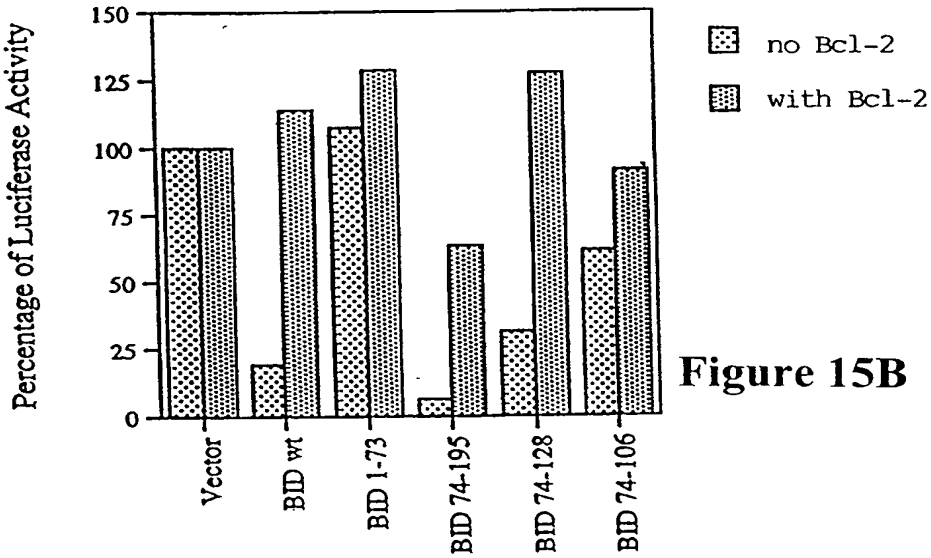


Figure 15B

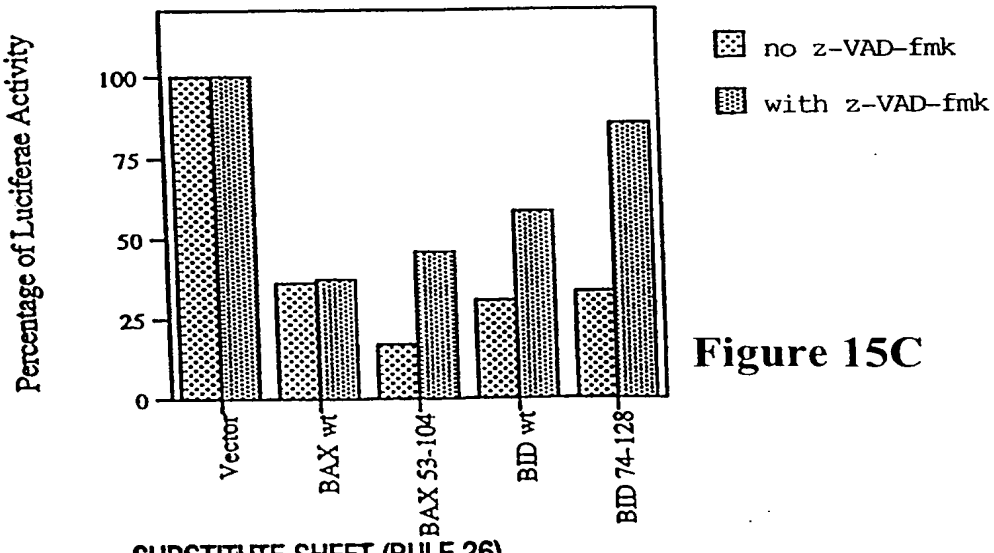


Figure 15C

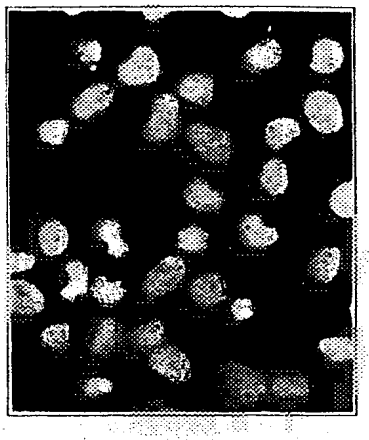


Figure 16A

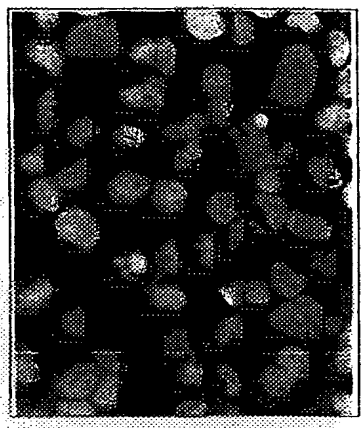


Figure 16B

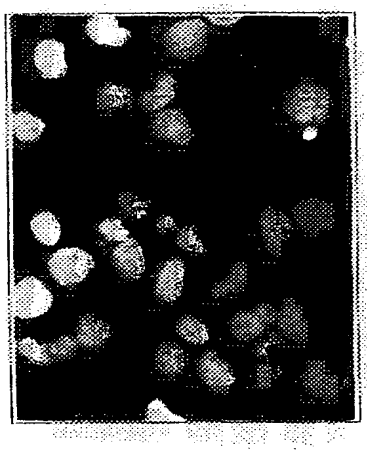


Figure 16C

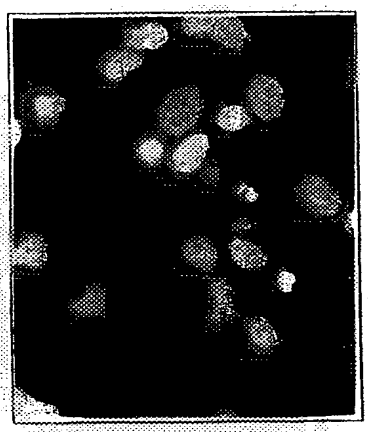


Figure 16D

TAT PEPTIDE	YGRKKRRQRRR	SEQ ID NO:55
BAX (53-86):	DASTKKLSECLKRIGDELDSNMELQRMIAAVDTD	SEQ ID NO:30
BAX (53-76):	DASTKKLSECLKRIGDELDSNMEL	SEQ ID NO:31
BAX (63-76)M:	DASTKKLSECELDLKRIGDSNMEL	SEQ ID NO:32
BAX (57-71):	KKLSECLKRIGDELD	SEQ ID NO:33
BAX (57-71)M:	KKLSECELDLKRIGD	SEQ ID NO:34
BAX (61-71):	ECLKRIGDELD	SEQ ID NO:35
BID (75-106):	DSESQEEIIHNIARHLAQIGDEMDHNIQPTLV	SEQ ID NO:36
BID (81-100):	EIIHNIARHLAQIGDEMDHN	SEQ ID NO:37
BID (81-100)M:	EIIHNIARHQIGDEMDLAHN	SEQ ID NO:38
BID (84-98):	HNIARHLAQIGDEMD	SEQ ID NO:39

Figure 17A

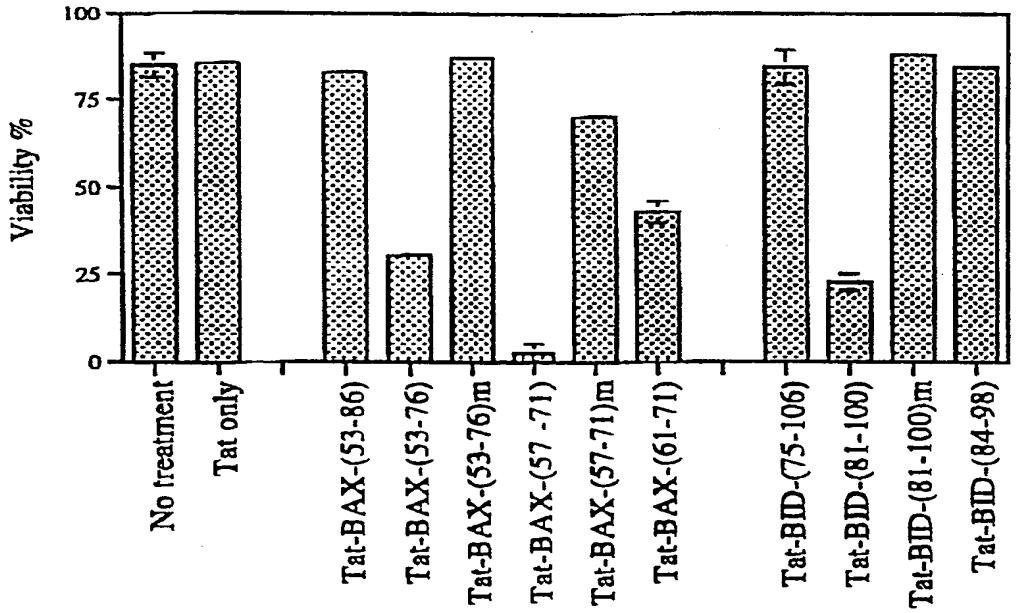


Figure 17B

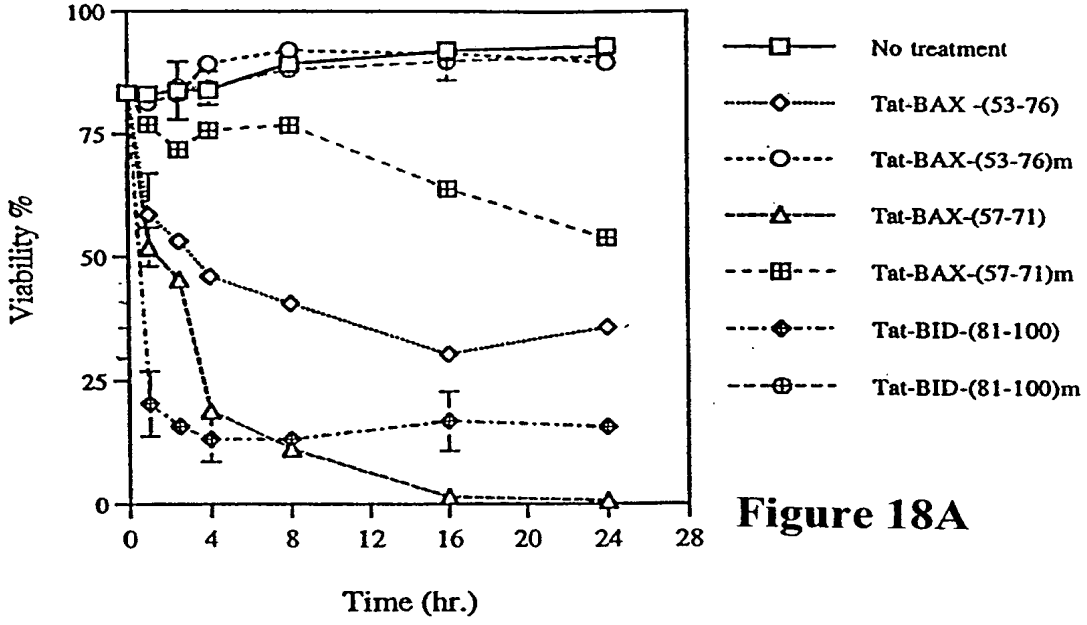


Figure 18A

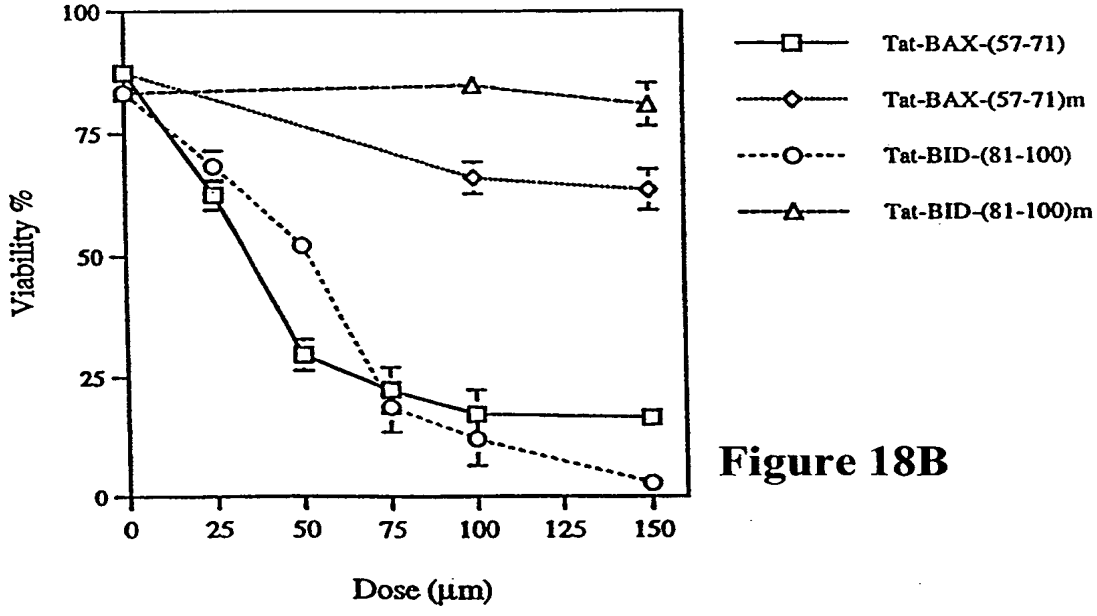


Figure 18B

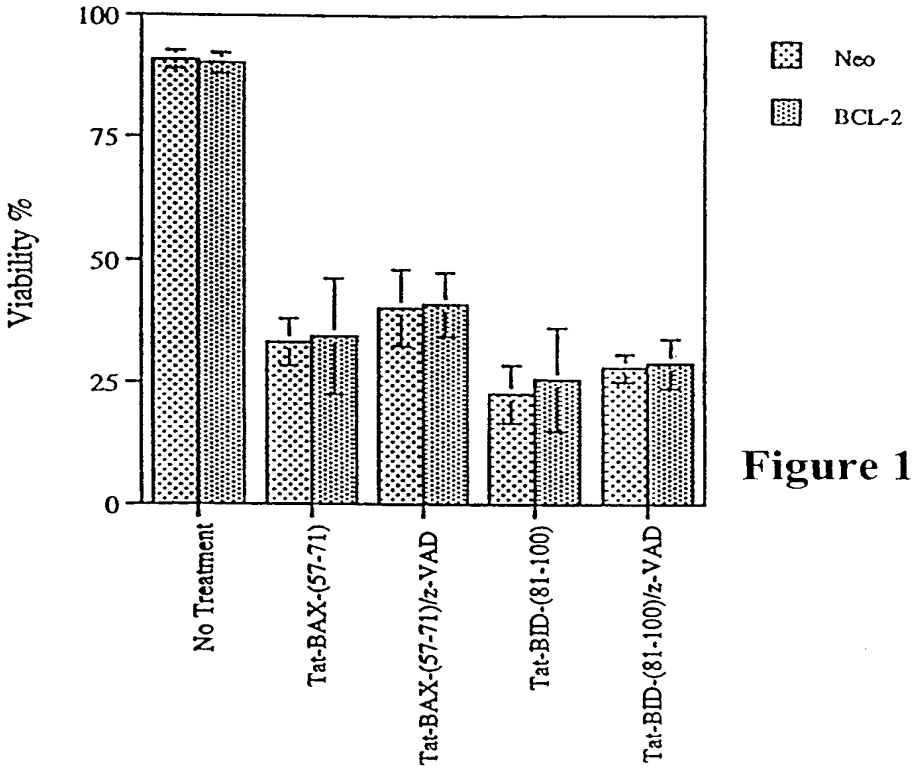


Figure 19A

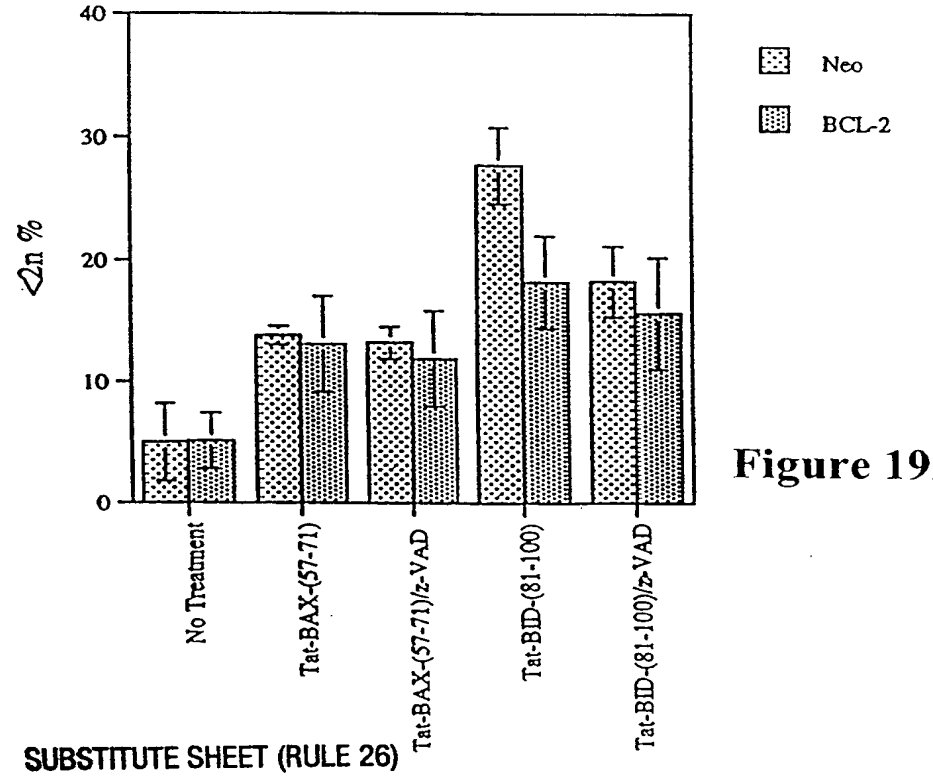


Figure 19B

Figure 20A

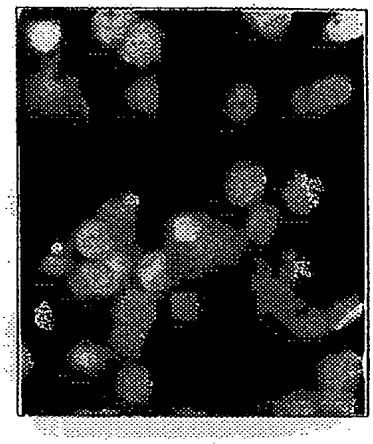


Figure 20B

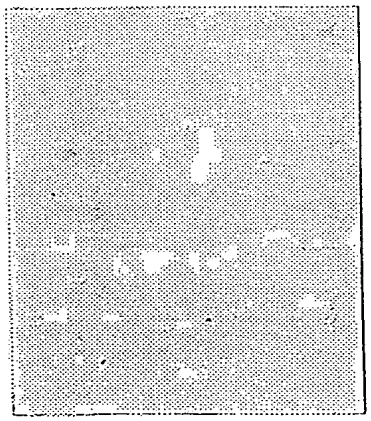


Figure 20C

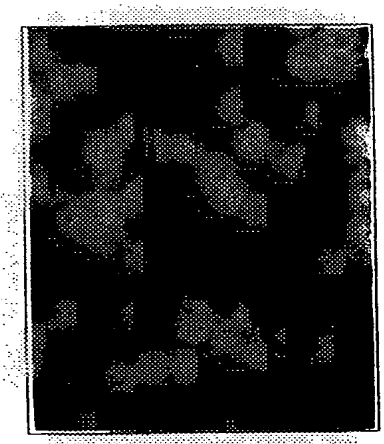


Figure 20D

Murine BAD and Partial Human BAD sequences

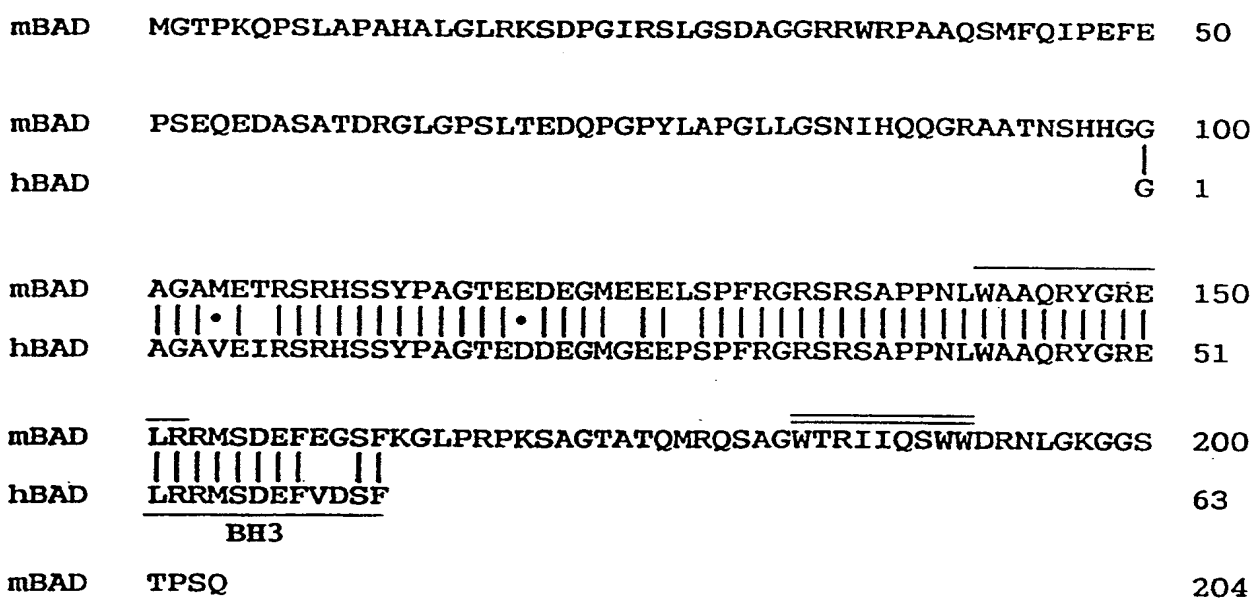


Figure 21A

Figure 21B

Murine BAK sequence

MASGQGPGPPKVGCDESPSPSEQQVAQDTEEVFRSYVFYLHQQEQETQGRPPANPEMDNLPLEPNSIL
GQVGRQLALIGDDINRRYDTEFQNLLEQLQPTAGNAYELFTKIASSLFKSGISWGRVVALLGFGYRLA
LYVYQRGLTGFLGQVTCFLADIILHHYIARWIAQRGGWVAALNLRRDPILTVMVIFGVVLLGQFVVHR
FFRS

Human BAK sequence

MASGQGPGPPRQECGEPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEMVTLPLQPS
STMGQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGINWGRVVALLGFGY
RLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQRGGWVAALNLGNGPILNVLVVLGVVLLGQFV
VRRFFKS

Figure 21C

Murine BAX sequence

MDGSGEQLGSGGPTSSEQIMKTGAFLLQGFIQDRAGRMAGETPELTLEQPPQDASTKKLSECLRRIGD
ELDSNMELQRMIADVDTDSPREVFFRVAADMFADGNFNWGRVVALFYFASKLVLKALCTKVPELIRTI
MGWTLDFLRERLLVWIQDQGGWEGLLSYFGTPTWQTVTIFVAGVLTASLTIWKKMG

Human BAX sequence

MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLSECLKRIGD
ELDSNMELQRMIAAVDTDSPREVFFRVAADMFSDGNFNWGRVVALFYFASKLVLKALCTKVPELIRTI
MGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGVLTASLTIWKKMG

```
huBid   - MDCEVNNGSSLRDECITNLLVFGFLQSCSDNSFRRELDALGHELPVLAPQ - 50
muBid   - MDSEVSNGSGLGAKHITDLLVFGFLQSSG--CTRQELEVLGRELPV-QAY - 47

huBid   - WEGY--DELQTDGNRSSHS-RLGRIEADSESQEDIIRNIARHLAQVGDSM - 97
muBid   - WEADLEDELQTDGSQASRSFNQGRIEPDSESQEEIIHNIARHLAQIGDEM - 97

huBid   - DRSIPPGLVNGLALQLRNTSRSEEDRNRDLATALEQLLQAYPRDMEKEKT -147
muBid   - DHNIQPTLVRQLAAQFMNGSLSEEDKRNCLAKALDEVKTAFPRDMENDKA -147

huBid   - MLVLALLLAKKVASHTPSLLRDVFHTTVNFINQNLRTYVRSLARNGMD   -195
muBid   - MLIMTMLLAKKVASHAPSLLRDVFHTTVNFINQNLFSYVRNLVRNEMD   -195
```

Figure 21D

Human BIK sequence

MSEVRPLSRDILMETLLYEQLLEPPTMEVLGMTDSEEDLDPMEDFDSLECMEGSDALALRLACIGDEMDVSLRAP
RLAQLSEVAMHSLGLAFIYDQTEDIRDVLRSFMDGFTTLKENIMRFWRSPNPGSWVSCEQVLLALLLLLALLLPL
LSGGLHLLLK

Figure 21E

Human BAD Partial Polynucleotide and Polypeptide Sequences

```
GGCGCTGGGGCTGTGGAGATCCGGAGTCGCCACAGCTCCTACCCCGCGGGGACGGAGGAC
60
 G  A  G  A  V  E  I  R  S  R  H  S  S  Y  P  A  G  T  E  D
20

GACGAAGGGATGGGGGAGGAGCCCAGCCCCTTTCGGGGCCGCTCGCGCTCGGCGCCCCCC
120
 D  E  G  M  G  E  E  P  S  P  F  R  G  R  S  R  S  A  P  P
40

AACCTCTGGGCAGCACAGCGCTATGGCCGCGAGCTCCGGAGGATGAGTGACGAGTTTGTG
180
 N  L  W  A  A  Q  R  Y  G  R  E  L  R  R  M  S  D  E  F  V
60

GACTCCTTT
189
 D  S  F
63
```

Figure 22A

Human BAK CDNA

```
   1 GAGGATCTAC AGGGGACAAG TAAAGGCTAC ATCCAGATGC CGGGAATGCA CTGACGCCCA
  61 TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC TGGGAGCAGC AGCCGCCAGC CCCTCGGACC
 121 TCCATCTCCA CCCTGCTGAG CCACCCGGGT TGGGCCAGGA TCCCGGCAGG CTGATCCCGT
 181 CCTCCACTGA GACCTGAAAA ATGGCTTCGG GGCAAGGCCC AGGTCCTCCC AGGCAGGAGT
 241 GCGGAGAGCC TGCCCTGCCC TCTGCTTCTG AGGAGCAGGT AGCCCAGGAC ACAGAGGAGG
 301 TTTTCCGCAG CTACGTTTTT TACCGCCATC AGCAGGAACA GGAGGCTGAA GGGGTGGCTG
 361 CCCCTGCCGA CCCAGAGATG GTCACCTTAC CTCTGCAACC TAGCAGCACC ATGGGGCAGG
 421 TGGGACGGCA GCTCGCCATC ATCGGGGACG ACATCAACCG ACGCTATGAC TCAGAGTTCC
 481 AGACCATGTT GCAGCACCTG CAGCCCACGG CAGAGAATGC CTATGAGTAC TTCACCAAGA
 541 TTGCCACCAG CCTGTTTGAG AGTGGCATCA ATTGGGGCCG TGTGGTGGCT CTTCTGGGCT
 601 TCGGCTACCG TCTGGCCCTA CACGTCTACC AGCATGGCCT GACTGGCTTC CTAGGCCAGG
 661 TGACCCGCTT CGTGGTCGAC TTCATGCTGC ATCACTGCAT TGCCCGGTGG ATTGCACAGA
 721 GGGGTGGCTG GGTGGCAGCC CTGAACTTGG GCAATGGTCC CATCCTGAAC GTGCTGGTGG
 781 TTCTGGGTGT GGTTCTGTTG GGCCAGTTTG TGGTACGAAG ATTCTTCAAA TCATGACTCC
 841 CAAGGGTGCC CTTTGGGTCC CGGTTCAGAC CCCTGCCTGG ACTTAAGCGA AGTCTTTGCC
 901 TTCTCTGTTC CCTTGCAGGG TCCCCCCTCA AGAGTACAGA AGCTTTAGCA AGTGTGCACT
 961 CCAGCTTCGG AGGCCCTGCG TGGGGGCCAG TCAGGCTGCA GAGGCACCTC AACATTGCAT
1021 GGTGCTAGTG CCCTCTCTCT GGGCCCAGGG CTGTGGCCGT CTCCTCCCTC AGCTCTCTGG
1081 GACCTCCTTA GCCCTGTCTG CTAGGCGCTG GGGAGACTGA TAACTTGGGG AGGCAAGAGA
1141 CTGGGAGCCA CTTCTCCCCA GAAAGTGTTT AACGGTTTTA GCTTTTTATA ATACCCTTGT
1201 GAGAGCCCAT TCCCACCATT CTACCTGAGG CCAGGACGTC TGGGGTGTGG GGATTGGTGG
1261 GTCTATGTTC CCCAGGATTC AGCTATTCTG GAAGATCAGC ACCCTAAGAG ATGGGACTAG
1321 GACCTGAGCC TGGTCCTGGC CGTCCCTAAG CATGTGTCCC AGGAGCAGGA CCTACTAGGA
1381 GAGGGGGGCC AAGGTCCTGC TCAACTCTAC CCCTGCTCCC ATTCCTCCCT CCGGCCATAC
1441 TGCCTTTGCA GTTGGACTCT CAGGGATTCT GGGCTTGGGG TGTGGGGTGG GGTGGAGTCG
1501 CAGACCAGAG CTGTCTGAAC TCACGTGTCA GAAGCCTCCA AGCCTGCCTC CCAAGGTCCT
1561 CTCAGTTCTC TCCCTTCCTC TCTCCTTATA GACACTTGCT CCCAACCCAT TCACTACAGG
1621 TGAAGGCTCT CACCCATCCC TGGGGGCCTT GGGTGAGTGG CCTGCTAAGG CTCCTCCTTG
1681 CCCAGACTAC AGGGCTTAGG ACTTGGTTTG TTATATCAGG GAAAAGGAGT AGGGAGTTCA
1741 TCTGGAGGGT TCTAAGTGGG AGAAGGACTA TCAACACCAC TAGGAATCCC AGAGGTGGAT
1801 CCTCCCTCAT GGCTCTGGCA CAGTGTAATC CAGGGGTGTA GATGGGGGAA CTGTGAATAC
1861 TTGAACTCTG TTCCCCCACC CTCCATGCTC CTCACCTGTC TAGGTCTCCT CAGGGTGGGG
1921 GGTGACAGTG CCTTCTCTAT TGGCACAGCC TAGGGTCTTG GGGGTCAGGG GGGAGAAGTT
1981 CTTGATTCAG CCAAATGCAG GGAGGGGAGG CAGATGGAGC CCATAGGCCA CCCCCTATCC
2041 TCTGAGTGTT TGGAAATAAA CTGTGCAATC CCCTCAAAAA AAAAACGGAG ATCC
```

Figure 22B

Human BAX sequence

```
  1 ATGGACGGGT CCGGGGAGCA GCCCAGAGGC GGGGGGCCCA CCAGCTCTGA GCAGATCATG
 61 AAGACAGGGG CCCTTTTGCT TCAGGGTTTC ATCCAGGATC GAGCAGGGCG AATGGGGGGG
121 GAGGCACCCG AGCTGGCCCT GGACCCGGTG CCTCAGGATG CGTCCACCAA GAAGCTGAGC
181 GAGTGTCTCA AGCGCATCGG GGACGAACTG GACAGTAACA TGGAGCTGCA GAGGATGATT
241 GCCGCCGTGG ACACAGACTC CCCCCGAGAG GTCTTTTTCC GAGTGGCAGC TGACATGTTT
301 TCTGACGGCA ACTTCAACTG GGGCCGGGTT GTCGCCCTTT TCTACTTTGC CAGCAAACTG
361 GTGCTCAAGG CCCTGTGCAC CAAGGTGCCG GAACTGATCA GAACCATCAT GGGCTGGACA
421 TTGGACTTCC TCCGGGAGCG GCTGTTGGGC TGGATCCAAG ACCAGGGTGG TTGGGACGGC
481 CTCCTCTCCT ACTTTGGGAC GCCCACGTGG CAGACCGTGA CCATCTTTGT GGCGGGAGTG
541 CTCACCGCCT CGCTCACCAT CTGGAAGAAG ATGGGCTGA
```

Human BID Sequence

Figure 22C

```
  1 ATGGACTGTG AGGTCAACAA CGGTTCCAGC CTCAGGGATG AGTGCATCAC
AAACCTACTG
 61 GTGTTTGGCT TCCTCCAAAG CTGTTCTGAC AACAGCTTCC GCAGAGAGCT
GGACGCACTG
121 GGCCACGAGC TGCCAGTGCT GGCTCCCCAG TGGGAGGGCT ACGATGAGCT
GCAGACTGAT
181 GGCAACCGCA GCAGCCACTC CCGCTTGGGA AGAATAGAGG CAGATTCTGA
AAGTCAAGAA
241 GACATCATCC GGAATATTGC CAGGCACCTC GCCCAGGTCG GGGACAGCAT
GGACCGTAGC
301 ATCCCTCCGG GCCTGGTGAA CGGCCTGGCC CTGCAGCTCA GGAACACCAG
CCGGTCGGAG
361 GAGGACCGGA ACAGGGACCT GGCCACTGCC CTGGAGCAGC TGCTGCAGGC
CTACCCTAGA
421 GACATGGAGA AGGAGAAGAC CATGCTGGTG CTGGCCCTGC TGCTGGCCAA
GAAGGTGGCC
481 AGTCACACGC CGTCCTTGGC TCCGTGATGT CTTTCACACA ACAGTAATTT
TATTAACCAG
541 AACCTACGCA CCTACGTGAG GAGCTTAGCC AGAAATGGGA TGGACTGA
```

Human BIK Sequence

Figure 22D

```
  1 CAGCATCGCC GCCGCCAGAG GAGAAATGTC TGAAGTAAGA CCCCTCTCCA GAGACATCTT
 61 GATGGAGACC CTCCTGTATG AGCAGCTCCT GGAACCCCCG ACCATGGAGG TTCTTGGCAT
121 GACTGACTCT GAAGAGGACC TGGACCCTAT GGAGGACTTC GATTCTTTGG AATGCATGGA
181 GGGCAGTGAC GCATTGGCCC TGCGGCTGGC CTGCATCGGG GACGAGATGG ACGTGAGCCT
241 CAGGGCCCCG CGCCTGGCCC AGCTCTCCGA GGTGGCCATG CACAGCCTGG GTCTGGCTTT
301 CATCTACGAC CAGACTGAGG ACATCAGGGA TGTTCTTAGA AGTTTCATGG ACGGTTTCAC
361 CACACTTAAG GAGAACATAA TGAGGTTCTG GAGATCCCCG AACCCCGGGT CCTGGGTGTC
421 CTGCGAACAG GTGCTGCTGG CGCTGCTGCT GCTGCTGGCG CTGCTGCTGC CGCTGCTCAG
481 CGGGGGCCTG CACCTGCTGC TCAAGTGAGC CCCCGGCGGC TCAGGCGTGG CTGGCCCCAC
541 CCCCATGACC ACTGCCCTGA GGTGGCGGCC TGCTGCTGTT ATCTTTTTAA CTGTTTTCTC
601 ATGATGCCTT TTATATTAAC CCCGTGATAG TGCTGGAACA CTGCTGAGGT TTTATACTCA
661 GGTTTTTTGT TTTTTTTTTA TTCCAGTTTT CGTTTTTTCT AAAAGATGAA TTCCTATGGC
721 TCTGCAATTG TCACCGGTTA ACTGTGGCCT GTGCCCAGGA AGAGCCATTC ACTCCTGCCC
781 CTGCCCACAC GGCAGGTAGC AGGGGGAGTG CTGGTCACAC CCCTGTGTGA TATGTGATGC
841 CCTCGGCAAA GAATCTACTG GAATAGATTC CGAGGAGCAG GAGTGCTCAA TAAAATGTTG
901 GTTTCCAGCA AAAAAAAAAA AAA
```

Figure 22E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/2; 530/300; 536/23.1, 23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/300; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DNA and amino acid databases
BH3 domain, SEQ ID NO: 1, 3, 5, 7, 9, 31, 33, 35, 37, 40, 55, Tat peptide, BCL-2 family, apoptosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,656,725 A (CHITTENDEN et al) 12 August 1997, see entire document.	1-4, 6, 8-11, 13-17, 19-21 --- 5, 7, 12, 18
X	BOYD et al. Bik, A Novel Death-Inducing Protein Shares a Distinct Sequence Motif with Bcl-2 Family Proteins and Interacts with Viral and Cellular Survival-Promoting Proteins. Oncogene. 1995, Vol. 11, pages 1921-1928, see entire document.	21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 DECEMBER 1998	Date of mailing of the international search report 26 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY A. JOHNSON Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHITTENDEN et al. A Conserved Domain in Bak, Distinct from BH1 and BH2, Mediates Cell Death and Protein Binding Functions. EMBO. 1995, Vol. 14, pages 5589-5596, see entire document.	21
X --- Y	WANG et al. BID: A Novel BH3 Domain-Only Death Agonist. Genes and Development. 1996, Vol. 10, pages 2859-2869, see entire document.	21 ----- 5, 12, 18
Y	US 5,652,122 A (FRANKEL et al) 29 July 1997, see abstract and SEQ ID NO:1.	7

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19765

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C07K 7/00, 14/00; C07H 21/04, 21/02; C12N 15/11; A61K 38/04, 38/16